Response of Indigenous Field Populations of *Fusarium graminearum* to Fungicide Application in Manitoba

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba in

partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Plant Science

University of Manitoba

Winnipeg

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ACKNOWLEDGEMENTS

I thank my academic supervisor, Dr Tom Graefenhan. He has provided technical support and professional guidance throughout my studies, and I am truly grateful to him for providing the opportunity to pursue this research project. I would also like to thank my co-advisor, Dr Anita Brûlé-Babel for her help and support and my committee members Dr Fouad Daayf and Dr Bruce Ford for their insightful questions and guidance towards my research project.

I thank the Canadian Grain Commission for the financial support throughout this project and the opportunity to conduct my research within the organisation. Without the support of a number of teams and individuals from this organisation, I could not have completed this research project. A special thanks is owed to the Microbiology team members, Tehreem, Niradha and Janice, for their warm friendship and technical help in the laboratory throughout my studies. I thank Dr Daniel Perry and members of the Variety Identification Team for their time and expertise that facilitated me completing the AFLP section of my research in their laboratory.

For her enthusiasm, kindness, and hard work, I extend a huge thank you to Maria Sachs. Maria helped in field trial spike sampling, fungal isolations, and played a key role in the development the fungicide sensitivity assay.

For granting me access to their field trials, and the provision of field trial disease assessments and weather data, I acknowledge Manitoba Agriculture and in particular a special thanks to Holly Derksen, for driving me around the province facilitating my field trial sampling.

Finally, I thank my husband Trevor for returning the favour, and of course our baby boy Toby.

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ABSTRACT

Allen, Sarah. M.Sc., The University of Manitoba, December, 2019. Response of indigenous field populations of *Fusarium graminearum* to fungicide application in Manitoba. Major Professor; Dr Tom Graefenhan.

Fusarium Head Blight (FHB) is an economically important fungal disease of wheat in North America that results in yield loss, reduced grain quality, and grain contamination due to production of mycotoxins by the primary causal species, *Fusarium graminearum*. Fungicide use is an important component of an integrated pest management strategy (IPM) for the control of FHB, although efficacies have been variable in the field. Field population characteristics, including high genetic diversity, suggest an ability of this pathogen to rapidly adapt to FHB management strategies. This study investigated the impact of fungicide application on the population dynamics of *F. graminearum* field populations. Across two spring wheat field trials located in Southern Manitoba, isolates were recovered from symptomatic spikes from untreated plots and plots that received a single fungicide application (a 1:1 prothioconazole and tebuconazole mixture) at two application timings. The effect of fungicide treatment on the genetic variation within field populations was assessed using Amplified fragment length polymorphism (AFLP) markers, which showed that fungicide treatment did not have a significant effect on the field population structure within a single growing season. The fungicide sensitivity level of each isolate to the same commercial fungicide product was assessed with an *in vitro* micro-titre plate method. No significant effect of field fungicide application on the

sensitivity levels of recovered isolates was observed, corroborating the genetic data. These data indicate that fungicide application is not significantly impacting *F. graminearum* field populations in Manitoba within a single growing season. The genetic data identified two subpopulations (previously described as NA1 and NA2) within each field trial that were strongly associated with trichothecene genotype. The fungicide sensitivity level of isolates from the NA2 subpopulation at one site was reduced in comparison to the NA1 subpopulation, but this difference was not observed in the other site. Different agronomic practices between the sites may indicate current management strategies are driving selection for populations with reduced sensitivity to triazole fungicides. Further studies exploring the impact of current management strategies on a wider geographical and temporal scale are required to better understand the population dynamics of *F. graminearum* to ensure the future sustainability of the management of this important fungal disease.

1 GENERAL INTRODUCTION

Fusarium head blight (FHB) is an important fungal disease of small grains and cereals worldwide. Since the 1900s, FHB has been a major crop disease in North America causing significant economic losses (Windels, 2000). *Fusarium graminearum* Schwabe (formerly telomorph: *Gibberella zeae* (Schwein) Petch) is the predominant causal species of FHB in wheat in Canada, particularly in the provinces of Saskatchewan and Manitoba (Clear and Patrick, 2010). The pathogen infects developing kernels leading to direct yield losses and a reduction in grain quality (Wegulo, 2012). It also produces secondary metabolites, known as trichothecenes, that are toxic to mammals, including humans (McCormick *et al.*, 2011). The presence of the typically chalky, shrivelled, disease-damaged kernels is a contributing factor in the downgrading of grain due to the impact on end use quality tolerance standards for the level of the trichothecene deoxynivalenol (DON) in grain shipments (Canadian Grain Commission, 2019). In 2016, a warm and wet summer was likely the cause of the highest FHB incidence in red spring wheat in Manitoba and Saskatchewan in recent history (Canadian Grain Commission, 2017b). The increase in the prevalence of FHB in the major wheat growing regions of Western Canada and in particular DON content in harvested grain is an ever increasing threat to a key crop commodity.

A major contributing factor to the economic losses caused by FHB is that there is no single effective approach to control the disease. Until the late 2000s, the majority of wheat varieties in Canada lacked FHB resistance with only a few moderately resistant varieties available.

Currently, there is just one hard red spring wheat variety (AAC Tenacious VB) and one winter wheat variety (Emerson) classed as FHB resistant in Manitoba, although these do not confer complete resistance (Seed Manitoba, 2017). Further to the lack of resistant hosts, chemical fungicides have demonstrated mixed results in the field against FHB on wheat. Currently, there is just one predominant chemical class registered for FHB control, the triazoles, and efficacies are limited to only suppressive activity (Gilbert and Tekauz, 2011). Trials using mixtures of triazole fungicides have shown that, although fungicides cannot eliminate the disease, they do have a role in limiting pathogen spread and mycotoxin accumulation (McMullen *et al.*, 2012). However, the efficacy of this approach varies within the chemical class and between replicated studies using the same active ingredient (Paul *et al.*, 2007).

High genetic diversity and large sizes of *F. graminearum* populations in North America provide a high evolutionary potential that can lead to adaptation of the pathogen to disease management practices, including the application of fungicides (Miedaner *et al.*, 2008). Changes in the population dynamic of *F. graminearum* across North America may be indicative of an emerging, more aggressive population. This has been demonstrated using molecular trichothecene chemotype profiling and population structure analysis using molecular markers (Ward *et al.*, 2008; Liang *et al.*, 2014; Kelly *et al.*, 2015). While the reason for such population change is not yet clear, there has been speculation on the role of a range of factors including greater fitness of the introduced population, climatic conditions, and agronomic factors such as the use of resistant wheat varieties and increased corn and soybean production (Ward *et al.*, 2008; Liang *et al.*, 2014). The impact of fungicides as a selective force on *F. graminearum* populations remains to be fully investigated.

Surveillance on the population dynamics of *F. graminearum* is vital for the sustainability and future development of pest management strategies for FHB control. This study aimed to assess the impact of triazole fungicide application on the genetic variation within natural field populations of *F. graminearum* in Manitoba that may be indicative of a change in the phenotypic variation in response to triazole fungicide use in spring wheat. This study addressed the main hypothesis that, even within a single growing season, triazole fungicide application acts as a selective force on genetically diverse *F. graminearum* field populations. This hypothesis was explored through two studies:

1. Amplified Fragment Length Polymorphism (AFLP) markers were used to test the hypothesis that differences in the genetic variation of natural field populations of *F. graminearum* sampled from plots with or without the application of a triazole fungicide product in Southern Manitoba would be observed. This study is presented in Chapter 3 of this thesis.
2. Phenotypic variation in the sensitivity of *F. graminearum* isolates to a triazole fungicide mixture product was assessed via an *in vitro* microplate study. This study tested the hypothesis that fungicide sensitivity levels of isolates recovered from fungicide treated plots would be greater than levels of isolates recovered from untreated plots. Consideration of the phenotypic data with the genetic data from the first study was used to test the hypothesis that groups of similar genotypes with distinct fungicide sensitivity levels would be identified among recovered isolates. This study is presented in Chapter 4 of this thesis.

2 LITERATURE REVIEW

2.1 Fusarium Head Blight of wheat

Fusarium Head Blight (FHB) is a fungal disease with a wide host range that poses a global threat to a number of key small grain crop species including wheat, oats, barley, and rice (Goswami and Kistler, 2004). Commonly known as scab, the disease has the potential to devastate the value of harvested wheat grain through several mechanisms: reduced yields, poor grain quality, and the contamination of grain with toxic secondary metabolites (mycotoxins) produced by certain *Fusarium* species (Windels, 2000; Pirgozliev *et al.*, 2003; Koga *et al.*, 2019).

From a global perspective, *F. graminearum* is the most common causal agent of FHB outbreaks in wheat. However, the disease can be associated with a number of species from the genus *Fusarium*, including *F. culmorum, F. poae, F. avenaceum, F. pseudograminearum,* and *F. sporotrichiodes.* The predominance of any of these species is largely dependent on the climatic conditions, host, and geography (Mesterházy *et al.*, 2003; Miedaner *et al.*, 2008). For example, in Australia *F. pseudograminearum* has been shown to contribute to FHB outbreaks alongside *F. graminearum* (Akinsanmi *et al.*, 2006). Changes in farming practices are hypothesised to have caused the replacement of *F. culmorum* by *F. graminearum* in the United Kingdom, which has coincided with an increased incidence of FHB (West *et al.*, 2012). *Fusarium graminearum* has always been the primary cause of FHB in wheat in North America (McMullen *et al.*, 2012). Annual surveys from wheat harvest samples in Western Canada have confirmed the predominance of *F. graminearum*, however, in lower disease years *F. avenaceum* has been the

dominant species in Saskatchewan and Alberta. In Manitoba, *F. graminearum* has consistently been the dominant species regardless of disease levels from harvested samples (Clear and Patrick, 2010).

Fusarium graminearum has been described as a species complex (O'Donnell *et al.*, 2000). Although morphologically indistinguishable, recent developments in molecular phylogenetic methods have expanded the *F. graminearum* species complex into distinctive individual species. O'Donnell *et al.*, (2000) first introduced a phylogenetic species recognition concept based on the sequence similarity of six single copy nuclear genes in thirty seven isolates from a global distribution. These isolates could be assigned across nine groups, creating eight novel species names, whilst retaining *F. graminearum senso stricto* for the most commonly occurring lineage seven. Further molecular analysis on a wider distribution and number of strains suggests the presence of sixteen distinct species to date (O'Donnell *et al.*, 2004; Starkey *et al.*, 2007; Sarver *et al.*, 2011). Although strongly supported by the molecular data, some authors have contested the newly defined species limits owing to their confirmation with alternative species concept definitions (Leslie and Bowden, 2008). Cross fertility studies have demonstrated that representatives from all nine lineages described by O'Donnell *et al.*, (2000) were able to cross with isolates from at least one other lineage, with all able to cross with lineage seven (Bowden *et al.*, 2006). Furthermore, validation of the molecular methods to define these lineages using a large number of isolates from Korea and South America demonstrated that further diversity limited the use of these tools for a wider geographical sample (Leslie *et al.*, 2007). Evidence supports that *Fusarium graminearum sensu stricto* is the principal *Fusarium graminearum* species complex lineage that causes FHB in wheat in North America (O'Donnell *et al.*, 2004;

Zeller *et al.*, 2004; Gale *et al.*, 2007; Starkey *et al.*, 2007). As such, *Fusarium graminearum sensu stricto* (hereafter referred to as *F. graminearum*) is the sole species under consideration in this research project.

2.2 Economic importance of FHB in Canada

Wheat is one of Canada's largest exported crop commodity, with 24 million tonnes of wheat exported in 2018 (Statistics Canada, 2019). Canada Western Red Spring (CWRS) wheat is globally renowned for its high protein and concomitant milling quality. Combined with other wheat varieties including durum wheat, exports on average contribute around $7 billion dollars annually to the Canadian economy (Agriculture and Agri-Food Canada, 2019). FHB poses a threat to Canadian wheat production and exports, both in terms of direct yield loss in the field and reduction in post-harvest grain quality that impacts downstream processing (Windels, 2000; Koga *et al.*, 2019). Since the 1990's, successive years of FHB epidemics in Western Canada and the US have threatened the value of this crop and thus created a need for investment into research to improve the management of this disease (Mcmullen *et al.*, 1997; Windels, 2000).

Due to the impact of *Fusarium* infection of kernels on the end use quality of grain, the visual presence of the disease (termed "*Fusarium* damage" (FUS DMG)) is used as one of many grading factors by the Canadian Grain Commission (Canadian Grain Commission, 2019). For example in CWRS wheat, the tolerance ranges from 0.3% FUS DMG for number one grade to 4% FUS DMG for feed grade (Canadian Grain Commission, 2019). Such tolerances mean that in a high disease year, growers can be substantially impacted by the grain price they can obtain, whist also causing concern for millers and bakers who are dependent on products that meet a

consistently high standard. For example, in 2016 the average Fusarium damaged kernels (FDK) incidence (samples with any presence of FDK) in harvested CWRS was 87% in Manitoba, and in excess of 90% in many districts. The severity (percentage of FDK per sample) was higher than 1% for many of these samples, which equated to a significantly reduced price obtained by the growers (Canadian Grain Commission, 2017a).

Fusarium graminearum is a producer of a number of mycotoxins, including deoxynivalenol (DON) which is toxic to mammals (Ferrigo *et al.*, 2016). Therefore, set tolerances for DON concentrations in harvested wheat have to be met depending on the end use of the grain. DON testing is not routinely performed on grain samples; instead FUS DMG is used as a proxy for DON levels. The level of FUS DMG in grain samples has repeatedly been demonstrated to be a good predictor for the amount of DON in samples (Paul *et al.*, 2005; Tittlemier *et al.*, 2019). However, weak correlations have been observed in some studies, possibly due to infection by different non-DON producing *Fusarium* species and environmental conditions affecting disease progression (de Rocquigny and Grenier, 2012; Amarasinghe *et al.*, 2013; Tittlemier *et al.*, 2019). Additional evidence of increased DON production in culture by an emerging *F. graminearum* chemotype has resulted in FUS DMG tolerances in grain to be tightened in Canada as of 2010 (Ward *et al.*, 2008; Western Grains Research Foundation, 2010).

2.3 *Fusarium graminearum* life cycle and infection biology

Fusarium graminearum is an hyphomycete that successfully overwinters in a saprophytic state on crop debris, which in turn acts as a primary inoculum source for the parasitic phase on wheat (Gilbert and Fernando, 2004). Although *F. graminearum* can cause root disease in wheat, the

infection on the spike is classed as a distinct disease and arises from aerially dispersed spores (Champeil *et al.,* 2004). Warm and wet weather encourages the mycelium in the debris to develop asexual spores (conidia) in sporodochia and sexual spores (ascospores) within fruiting bodies known as perithecia (Goswami and Kistler, 2004). This often coincides with the anthesis stage of cereal crops, which in turn creates natural entry points for spores that land and germinate on the wheat spikes (Brown *et al.,* 2010). The ascospores are discharged from perithecia and can travel large distances by wind when wet episodes are followed by drier periods. The conidia are spread on a more local scale by rain-splash (Parry *et al.,* 1995).

Due to the short susceptibility window of wheat to *F. graminearum,* FHB is often described as a monocyclic disease (Trail, 2009). The importance of either ascospores or conidia in the primary infection remains unclear. A study conducted in New York state by Del Ponte *et al.,* (2003) provided evidence that ascospores external to the local field were important contributors to the primary inoculum of FHB in winter wheat. Other studies have shown a rapid decline in ascospore concentrations from an initial source, suggesting that local spore dispersal (either from ascospores or conidia) was the predominant inoculum source (Gilbert and Fernando, 2004). Furthermore, crop rotation and crop residue management has long been a method for reducing the risk of FHB, providing further evidence of the primary role of field level inoculum sources in the FHB disease (Gilbert and Tekauz, 2011; Gilbert and Haber, 2013; Shah *et al.,* 2018). Mixed efficacies in cropping practices to control FHB and contrasting data from spore dispersal studies suggest the primary source of inoculum for FHB is affected by a number of variables, which in turn determine the disease levels spatially and temporally.

It has long been acknowledged that FHB initiates as a floral infection of wheat spikes. The flowering stages of the crop appears to provide tissues (palea, glumes and lemna) that are susceptible to spore penetration through stomata, but also by direct penetration using an array of plant cell wall degrading enzymes (Parry *et al.*, 1995; Pritsch *et al.*, 2000; Brown *et al.*, 2010; Walter *et al.*, 2010). Once the germinating spore enters these spikelet tissues, intercellular hyphae spread in the apoplast. This stage can be described as a biotrophic phase, as no host cell damage occurs (Walter *et al.*, 2010). Host cell death occurs prior to successful intracellular colonisation of the spikelet and thus the nutritional mode of the pathogen at this phase can be described as necrotrophic (Brown *et al.*, 2010; Walter *et al.*, 2010).

Complex molecular interactions between the host and pathogen during infection and colonization have been elucidated in numerous studies, as summarized by Walter *et al.*, (2010). Host defence responses were observed as early as six hours after inoculation, with a peak at 36 to 48 hours after inoculation (Pritsch *et al.*, 2000). Recent studies on the recombination events in *F. graminearum* and genomic diversity across multiple *F. graminearum* isolates have revealed recombination and diversity hotspots in areas involved in host virulence (Laurent *et al.*, 2017, 2018). While such studies can provide possible targets for disease control, they demonstrate the functional redundancy within the array of pathogen effectors with the potential for rapid evolution, and thus the challenge of controlling the pathogen with a single approach.

The necrotrophic infection phase leads to the typical spikelet bleaching symptoms of FHB. Once a single spikelet has been colonized, the disease can progress throughout the spike, increasing the severity of the disease. The severity is influenced by a number of factors including the

aggressiveness of the *F. graminearum* isolate, the susceptibility of the host, and the environmental conditions (Buerstmayer *et al.*, 2009). Brown *et al.*, (2010) showed microscopically how the invading hyphal front moves first through the cortex and then through the vasculature of the rachis and into neighbouring spikelets in just a few days after initial inoculation. The hyphal colonization of the vasculature may also lead to premature senescence of uninfected tissue above the point of inoculation due to interruption of the passage of nutrients and water through the xylem (Savard *et al.*, 2000; Brown *et al.*, 2010)

The damage to the developing kernels in the necrotrophic colonisation phase is when crop losses and reduction in grain quality occurs (Wegulo, 2012). During the infection cycle, *F graminearum* produces a range of enzymes to facilitate host cell breakdown and nutrient acquisition (Brown *et al.*, 2017). These include secreted proteases, that have been shown to break down gluten proteins in the endosperm of the kernel, resulting in a reduction in the bread making quality of the flour milled from infected grain (Koga *et al.*, 2019). It is also during this stage that the biosynthesis of the predominant *F. graminearum* mycotoxin, DON, increases. DON is a proposed virulence factor contributing to the pathogens ability to overcome an array of host defences including reactive oxygen species signalling and salicylic acid mediated processes (Audenaert *et al.*, 2013). Knock-out mutants deficient in DON production supported the role of this mycotoxin in the further colonisation of neighbouring spikelets via the rachis (Maier *et al.*, 2006). Therefore, there is often a correlation between visual disease symptoms and DON content in grain. However, the relationship between these two variables is influenced by other factors including host and pathogen genotype, agricultural practices, and the environment (Maier *et al.*, 2006; Wegulo, 2012; Audenaert *et al.*, 2013).

2.4 *Fusarium graminearum* mycotoxins

Fusarium species produce an array of mycotoxins including, but not limited to, zearalenone, fumonisins and trichothecenes (Ferrigo *et al.*, 2016). *Fusarium graminearum* most commonly produces trichothecenes which are derived from terpene and for which the biosynthetic pathways have been well characterized (Proctor *et al.*, 2009; McCormick *et al.*, 2011). Trichothecenes are rapidly absorbed when ingested by mammals where they produce harmful effects including vomiting, anorexia, and even death at high doses (Ferrigo *et al.*, 2016). Many countries have set concentration limits in cereal products intended for human and animal consumption (Ferrigo *et al.*, 2016; Codex Alimentarius Commission, 2019).

The trichothecenes encompass a large family of molecules that share a tricyclic 12,13-epoxytrichothec-9-ene core structure and can be split into four groups: Types A, B, C, and D (McCormick *et al.*, 2011). *Fusarium* species produce type A and type B trichothecenes which differ in the functional groups in the C-8 position (McCormick *et al.*, 2011; Ferrigo *et al.*, 2016). *Fusarium graminearum* produces type B trichothecenes which include nivalenol (NIV), DON and derivatives, 3-acetyldeoxynivalenol (3ADON) and, 15-acetyldeoxynivalenol (15ADON) (McCormick *et al.*, 2011). *Fusarium graminearum* isolates typically produce either NIV, DON and 3ADON or DON and 15ADON, which in turn are referred to as the chemotypes NIV, 3ADON and 15ADON respectively (McCormick *et al.*, 2011; Pasquali and Migheli, 2014). Isolates that produce a type A like trichothecene, termed NX-2 chemotypes, have been found recently in North America, although these appear at low frequencies in sampled isolates (Liang *et al.*, 2014; Kelly *et al.*, 2015; Varga *et al.*, 2015).

Thorough studies on the biosynthetic pathways and genetic basis of trichothecene biosynthesis have provided an in depth understanding of the mutations underpinning the different chemotypes in *F. graminearum* and other *Fusarium* species. Three separate loci are involved in trichothecene biosynthesis: the *TRI* cluster, the two gene *TRI1-TRI16* locus, and the *TRI101* locus (McCormick *et al.*, 2011). The *TRI* cluster includes twelve *TRI* genes, including *TRI7* and *TRI13.* In NIV producing strains, *TRI7* and *TRI13* are functional, whereas insertion and deletion mutations in these genes are found in DON producing strains which removes their functionality (Brown *et al.*, 2002; Lee *et al.*, 2002). Sequence differences in the *TRI8* gene of the *TRI* cluster locus have been shown to encode two different enzymes that determine the 15ADON and 3ADON chemotypes (Alexander *et al.*, 2011). NX-2 chemotypes have the 3ADON allele in the *TRI8* gene, but polymorphisms in the *TRI1* gene are associated with NX-2 producers (Liang *et al.*, 2014).

As discussed, trichothecenes are a concern from a food safety perspective and in terms of disease development, due to their role as virulence factors. In some regions of North America, the emergence of 3ADON producing *F. graminearum* isolates, in a historical landscape of 15ADON producing isolates, has given rise to numerous comparison studies on the aggressiveness and DON production of these two chemotypes. Laboratory studies indicate that the 3ADON producing isolates are more aggressive, produce more DON in culture, and have greater phytotoxic potential than 15ADON producers (Ward *et al.*, 2002; Desjardins *et al.*, 2007; Puri and Zhong, 2010). Studies *in planta* are less conclusive. Ward *et al.*, (2008) showed that from subsets of 3ADON and 15ADON *F. graminearum* isolates, FHB severity in a susceptible and a moderately resistant wheat variety did not differ with chemotype used for spray

inoculation. However, in a susceptible variety, the DON content was significantly higher in spikes inoculated with 3ADON isolates. A similar glasshouse study demonstrated that 3ADON chemotypes produced more DON and were more aggressive than 15ADON chemotypes on a susceptible and a moderately resistant wheat variety, however, this was not observed on a third resistant wheat variety (Puri and Zhong, 2010). A large field level study using six spring wheat varieties across multiple environments in Canada and Germany concurred with previous studies, reporting minimal differences in aggressiveness among 15ADON and 3ADON chemotypes across host genotypes (von der Ohe *et al.*, 2010). However, 3ADON isolates produced more DON content in five out of six of the wheat varieties that included moderately resistant varieties. These studies indicate that while FHB disease can be managed with the use of resistant varieties, the control of DON content may be less successful with changing *F. graminearum* population dynamics.

2.5 FHB management strategies

FHB is a complex disease that cannot be controlled with an individual strategy. Successful management of this disease requires an integrated pest management (IPM) approach by the grower. Studies on the effects of multiple strategies have increased in recent years, with much focus on the combination of genetic resistance in the host and chemical fungicide use (McMullen *et al.*, 2008; Willyerd *et al.*, 2012). In the following sections, consideration will be given to the key components of the FHB IPM approach.

2.5.1 Cultural control

Cultural management strategies for the control of FHB are aimed at reducing the inoculum reservoir in the field. As discussed in Section 2.3, local inoculum sources from previous crop residues provide a primary source of inoculum for FHB. Crop rotation and residue management are therefore recommended to prevent the build-up of diseased material in the field. However, these methods have shown to be both limited and variable in their efficacies, and thus are used as a complement to other management practices.

Tillage practices can chop up and bury crop residues which can speed up their decomposition and therefore reduce the nutrient source for the saprophytic life stage of FHB species (Miller *et al.*, 1998; Fernandez *et al.*, 2005). However, the efficacy of tillage alone has been shown to have minimal effect on FHB disease control. Conventional tillage compared to no tillage in a three year wheat-corn-soybean rotation study showed that tillage significantly reduced the prevalence of an artificially introduced strain (Miller *et al.*, 1998). However, this did not correlate with significant differences in FHB incidence and severity between the two tillage treatments (Miller *et al.*, 1998). Similar results have been reported from a field study with natural inoculum, where the environmental conditions appeared to override any effect of tillage practices (Lori *et al.*, 2009). In a multi-year survey of fields in Ontario, tillage was shown to have minimal effect on DON concentration in harvested wheat grain under moderate disease levels, although some differences were observed following an epidemic year (Schaafsma *et al.*, 2001). However, it should also be considered that conventional tillage is rarely used in the prairies due to a desire to preserve soil moisture and prevent soil erosion (Gilbert and Tekauz, 2011). Combined with a

lack of evidence for real-world control of FHB, tillage is not considered to be a primary FHB management strategy.

Crop rotation has been shown to be a more effective tool for FHB management, in comparison to tillage. Studies consistently show that alternating wheat with a non-host for FHB provide significant reductions in disease, with pulse crops providing the best results as a break crop (Gilbert and Tekauz, 2011). For example, in a field plot study, FHB disease levels were highest following corn than wheat, and lowest when soybean was the previous crop (Dill-Macky and Jones, 2000). Interestingly, a corn-wheat crop sequence has been shown to result in higher FHB disease levels than a wheat-wheat sequence (Fernandez *et al.*, 2005). A study of colonisation of a diverse range of plant species by *F. graminearum* showed that inoculum levels were highest on wheat and barley residues, however corn residues were slower to decompose and so retained inoculum levels for longer (Pereyra and Dill-Macky, 2008). Furthermore, tillage was shown to have a significant effect on FHB incidence and severity when corn was grown the previous year, but not when soybean was grown previously (Dill-Macky and Jones, 2000). This suggests that corn leaves substantial levels of inoculum in the field by way of slow to decompose residues, and as such avoiding planting wheat directly after corn is an important factor in FHB control.

Fusarium graminearum has also been isolated from asymptomatic inflorescences and seed of wild grasses in North America (Inch and Gilbert, 2003; Lofgren *et al.*, 2018). The role of wild grasses as an inoculum source in crop species is unknown, however Pereyra and Dill-Macky, (2008) demonstrated in-field survival of *F. graminearum* on native grass residues in Uruguay and the production of ascospores on residues when incubated in an artificial environment.

However, colonisation and ascospore production were much lower relative to crop residues, leading the authors to speculate that native grasses may play a minor role as an inoculum source (Pereyra and Dill-Macky, 2008). However, the saprophytic life stage on native grasses may allow for survival between susceptible crops, although the importance of this stage in disease outbreaks remains unclear (Inch and Gilbert, 2003).

2.5.2 FHB resistance in wheat

A high level of disease resistance in crops is often attributed to a single major resistance gene. This is known as qualitative resistance and is often driven by the plant's recognition of the invading pathogen via the protein encoded by the resistance gene. This is followed by an induction of a defence response by the plant that cannot be overcome by the pathogen (Nelson *et al.*, 2018). In the case of the wheat versus *F. graminearum* pathosystem, such major resistance genes have been elusive and thus resistance to date has been limited to quantitative disease resistance driven by multiple genes (Gilbert and Haber, 2013). Due to the complexities of breeding quantitative disease resistance, the development of FHB resistant wheat varieties has been met with only partial success to date.

FHB resistance breeding has focussed on identifying groups of linked genes responsible for reaction to FHB, known as quantitative trait loci (QTL). QTLs have been discovered on every wheat chromosome and multiple major QTLs have been found in winter and spring wheat varieties (Buerstmayer *et al.*, 2009; Gilbert and Haber, 2013). Major sources of resistant germplasm are from Asian varieties and wild species. The most commonly used QTLs come

from the Sumai 3 variety and are *Fhb1*, *Fhb2* and *Qfhs.ifa-5A* with *Fhb1* used in most resistant breeding programs in North America (Steiner *et al.*, 2017).

With the exception of *Fhb1*, challenges in identifying markers for most QTLs have limited marker assisted breeding programmes (Steiner *et al.*, 2017). Most resistant varieties have arisen from successive screening for resistance in phenotypic trials, however, these are both time consuming and expensive (Gilbert and Haber, 2013). For instance, the genetic causes for the high FHB resistance rated hard red winter wheat variety Emerson are yet to be determined (Graf *et al.*, 2013). Furthermore, linkage and pleiotropic effects from QTLs can have deleterious effects on other desired traits such as plant height, grain filling, and protein content (Bai and Shaner, 2004; McCartney *et al.*, 2007). This can create reluctance by growers to embrace the FHB resistant varieties in favour of higher yielding susceptible varieties (Bai and Shaner, 2004). These challenges in breeding for FHB resistance in wheat and a lack of varieties with 100% FHB resistance has led to a focus on the importance of selecting suitable varieties in combination with other management tools, *i.e.* an IPM approach.

2.5.3 Chemical Fungicides

2.5.3.1 Efficacy of fungicides in the control of FHB

Synthetic fungicides are widely recognized to be a vital component of FHB management. In Canada, as is the case for most countries, the triazole chemical class remains the sole class of fungicides used against FHB. The large strobilurin fungicide class has been shown to be ineffective for controlling FHB incidence and DON and is therefore not recommended for FHB

control. In some studies, their use has even been shown to increase DON content in grain (Simpson *et al.*, 2001; Caldwell *et al.*, 2017). Registered active ingredients in Manitoba within the triazole class for use against FHB are metconazole, tebuconazole and prothioconazole. Prothioconazole and tebuconazole are offered as both solo products and as a mixture product. These products are described as offering suppressive activity only, *i.e.* they do not fully control this disease (Manitoba Agriculture, 2018). The application of these fungicides is recommended at between 20% and 50% anthesis, which is when FHB infection is most likely to occur. As such, this only affords growers a relatively small window of opportunity for application.

Although the triazole fungicides have limited activity against FHB, their use has been widely established as being economically valuable due to their efficacies at reducing FHB severity and/or DON levels in wheat. This has been demonstrated by replicated field trials conducted in the U.S. across multiple years and locations coordinated by the U.S. Wheat and Barley Scab Initiative (USWBSI) using standardised protocols, enabling inter-trial comparisons (McMullen *et al.*, 2012). A meta-analysis by Paul *et al.*, (2008) using data from the USWBSI trials suggests that the product mixture of prothioconazole and tebuconazole had the highest efficacy against FHB severity, whereas metconazole was superior at reducing DON content. However, differences between fungicides were small and a high level of variability between trials was observed (Paul *et al.*, 2008). Therefore, although general trends on which products provide superior efficacy can be determined by such studies, it is clear that other factors contribute to the field efficacy of fungicides against FHB.

The studies used in the meta-analysis by (Paul *et al.*, 2008) all used a single application of fungicide treatment at anthesis. Although fungicide product labels recommend spraying at 20% to 50% anthesis, there is evidence that application five to seven days later can provide the same level and even improved reduction in FHB incidence and DON content. Studies by Tateishi *et al.*, (2014) and Yoshida *et al.*, (2012) demonstrate that spraying fungicide treatment at the recommended timing of early anthesis reduces visual symptoms compared to later application. However, DON content was lower when application was later than early anthesis. Field trials in the U.S Midwest showed later fungicide timing (up to six days post 50% anthesis) was as effective at controlling visual disease symptoms and DON as application at 50% anthesis (D'Angelo *et al.*, 2014) . The potential for more flexibility in fungicide application is a benefit to the grower; however legal pre-harvest intervals to control pesticide residues on grain are a limitation to late application.

2.5.3.2 Integrated pest management of FHB with resistant varieties and fungicides

The interaction between fungicide treatment and wheat variety on the control of FHB has been extensively researched. A meta-analysis using data from USWBSI trials demonstrated that fungicide treatment (prothioconazole plus tebuconazole) and host resistance has an additive effect (Willyerd *et al.*, 2012). Although a clear overall trend was demonstrated, variability in FHB severity between trials was also observed. However, DON levels were less variable across sites. The combination of fungicide application and a moderately resistant wheat variety showed less variation than combinations of fungicide application with moderately susceptible and susceptible varieties (Willyerd *et al.*, 2012). A two year spring wheat field study conducted in Manitoba by Amarasinghe *et al.*, (2013) investigated the efficacies of triazole fungicides on a

moderately resistant variety and susceptible variety. Trials were artificially inoculated with four *F. graminearum* isolates and assessed for %FDK, FHB index (a formula encompassing fusarium incidence and severity in the field) and DON. While overall fungicide use, variety, and the interaction of both factors had significant effects on the %FDK and FHB index in both years, there was poor correlation with DON concentration. Individually, fungicide and variety failed to significantly reduce DON over non-fungicide treated plots in both years. However, there was a significant interaction between the two factors on the amount of DON in one year (Amarasinghe *et al.*, 2013). While this study would support a combined strategy of fungicide application and variety selection for improved control of FHB, it also demonstrates unpredictability with efficacies with respect to DON concentration control.

In summary, the existing body of literature demonstrates the importance of integrating multiple management strategies to control FHB disease progression and DON accumulation in wheat. However, there is clearly a complex interaction between the main strategies of fungicide use and variety selection which is yet to be fully understood. Fungicides remain high on the list of tools to control FHB in Manitoba, where the wet summer climate creates favourable conditions for FHB. Fully understanding the causal factors of the variation in the efficacies of fungicides is essential to ensuring the economic viability of their use.

2.5.3.3 Triazole fungicide resistance in *F. graminearum*

Widespread use of a chemical fungicide always warrants monitoring of the development of resistance to the chemical class by a pathogen (Ma and Michailides, 2005). The DMI class, which includes triazoles, has been used extensively in a number of crops for a range of fungal

diseases since they were introduced in the 1970's, and reductions in sensitivity to this class have been observed for many phytopathogenic fungal species (Ma and Michailides, 2005; Yin *et al.*, 2009; Fan *et al.*, 2013). Multiple mechanisms of resistance to DMI's have been identified, and often combinations of these mechanisms within a single isolate have been identified (Ma and Michailides, 2005; Cools *et al.*, 2013). This polygenic nature of DMI resistance has made for a complex landscape of cross-resistance to different active ingredients within the DMI fungicide class among fungal isolates (Ma and Michailides, 2005; Cools *et al.*, 2013; Fan *et al.*, 2013).

There is evidence that populations of *F. graminearum* are, like other phytopathogenic fungal species, able to adapt to the use of triazole fungicides. Klix *et al.*, (2007) demonstrated that since the introduction of four single triazoles, the sensitivity of sampled isolates from Germany to each active ingredient has gradually declined over time; however, newer fungicides still demonstrated good levels of efficacy. A temporal comparison of the *in vitro* sensitivity of field isolates to tebuconazole in China demonstrated a sensitivity shift by a factor of 1.53 from 2000 to 2012 (Sun *et al.*, 2014). Field isolates with clear resistance to DMIs have also been recovered in China and North America, demonstrating an extreme sensitivity shift, although frequencies were low (Yin *et al.*, 2009; Spolti *et al.*, 2014). To date the mechanism(s) for DMI resistance in *F. graminearum* remain unknown.

Triazole fungicides target the enzyme p450 sterol 14α-demethylase which disrupts ergosterol biosynthesis. In most species this is encoded by a single gene, *CYP51,* and point mutations in this gene and / or overexpression of the gene has been attributed to triazole resistance in many fungal pathogens (Ma and Michailides, 2005; Cools *et al.*, 2013). *Fusarium graminearum* is rare

in that it has three *CYP51* paralogs (*CYP51A*, *CYP51B* and *CYP51C*) (Fan *et al.*, 2013). Mutagenesis studies have elucidated a diverse range of functions of the *CYP51* genes in *F. graminearum*, including sterol biosynthesis, virulence and ascospore production (Fan *et al.*, 2013). *CYP51A* expression was shown to be induced upon *in vitro* triazole treatment and deletion mutants increased sensitivity to triazole fungicides, although cross-resistance was incomplete (Liu *et al.*, 2011; Fan *et al.*, 2013). While the deletion mutants in the other two *CYP51* genes also affected the triazole sensitivity, results were mixed and it was proposed that *CYP51A* was the primary determinant of this trait (Liu *et al.*, 2011; Fan *et al.*, 2013). In contrast to these laboratory studies, characterisation of the tebuconazole resistant *F. graminearum* field isolates from China showed that neither overexpression, nor point mutations in any of the *CYP51* paralogs was associated with the resistance (Yin *et al.*, 2009).

Upregulation of ATP-binding cassette (ABC) transporters have also been implicated in triazole resistance in other pathogens, facilitating the efflux of the toxic molecules from the cell (Ma and Michailides, 2005; Cools *et al.*, 2013). *Fusarium graminearum* has 62 putative ABC transporters, and transcriptional upregulation of a number of these genes was observed upon exposure of *F. graminearum* isolates to tebuconazole (Liu *et al.*, 2010; Becher *et al.*, 2011; King *et al.*, 2015). Functional studies on a number of these genes have confirmed a role in sensitivity of *F. graminearum* to DMIs (Ammar *et al.*, 2013; Qi *et al.*, 2018). While this mechanism of resistance has not been confirmed in DMI resistant *F. graminearum* field isolates, the observed cross-resistance to other fungicide class's, points to a potential general efflux method (Yin *et al.*, 2009). Furthermore, the role of an ABC transporter gene in triazole sensitivity has been confirmed in field isolates of the closely related species, *Fusarium culmorum* (Hellin, 2018)

The indication that sensitivity to triazoles is controlled by multiple genes in *F. graminearum*, combined with observed gradual sensitivity shifts in *F. graminearum* populations, suggests that a sudden shift in the triazole sensitivity of populations in North America is unlikely. However, a sparsity of new chemical classes to control FHB, and therefore, a reliance on the DMI class warrants close observation of potential adaptation of *F. graminearum* populations to this technology.

2.6 *Fusarium graminearum* population genetics and dynamic in North America

The widespread occurrence of FHB has prompted considerable research into the epidemiology, evolutionary history, and control of this pathogen. Of particular concern is the ability of the pathogen to rapidly adapt and proliferate in both existing and new environments. *Fusarium graminearum* is a haploid, homothallic fungus that produces ascospores within a perithecium. Although homothallic reproduction may be a primary method for *F. graminearum* to produce durable inbred ascospores, the potential for outcrossing has been demonstrated in the laboratory (Bowden and Leslie, 1999). As discussed in Section 2.3, ascospores have the potential to travel long distances and may form an important primary inoculum source. The combination of a sexual life cycle with an ability for outcrossing and large geographical dispersal mechanisms are ideal biological characteristics for rapid evolution, and the subsequent widespread dissemination of novel traits (McDonald and Linde, 2002). The duality of a saprophytic and parasitic life cycle combined with the ability to infect a wide range of hosts is likely to result in balancing selection for a range of fitness traits, contributing to a genetically diverse population, and thus further enhancing its adaptive ability (Miedaner *et al.*, 2008). Furthermore, as *F. graminearum* can

overwinter in grain, global grain movement has the potential to introduce novel strains into new geographical areas where their potential effects on disease outbreaks and severity are unknown (Ward *et al.*, 2008).

Studies have utilised genetic marker technology to assess the genetic diversity and differentiation of *F. graminearum* populations on wheat at a range of geographical scales. In Canada, the use of Random Amplified Polymorphic DNA (RAPD) markers demonstrated high genetic and genotypic diversity with no clustering in sampled isolates from Quebec and Ontario (Dusabenyagasani *et al.*, 1999). Inter-sequence simple repeat markers revealed a similar picture in the three western Canadian prairie provinces, with the majority (97.23%) of the molecular variation observed within provincial populations (Mishra *et al.*, 2004). Zeller *et al.*, (2004) used AFLP markers on seven field populations distributed throughout the central and eastern United States, high genotypic diversity was observed in all populations and all populations were genetically similar. Zeller *et al.*, (2003) showed that the majority of the molecular variation could occur within areas as small as $0.25m^2$ quadrats within a single field. These studies pointed to a genetically diverse *F. graminearum* population in North America, with low levels of genetic differentiation across the continent.

Contrary to the above mentioned studies, there is evidence of a change in the population dynamic of *F. graminearum* across North America that may be indicative of an emerging, more aggressive population. This has been demonstrated using molecular mycotoxin chemotype profiling and population structure analysis (Ward *et al.*, 2008; Liang *et al.*, 2014). While the 15ADON chemotype was the predominant chemotype, surveys suggest a clear shift from

15ADON to 3ADON chemotypes in Eastern Canada provinces (Ward *et al.*, 2008), while Ontario has predominantly 15ADON chemotypes (Tamburic-Ilincic *et al.*, 2015). In Western Canada and the Midwestern US states, the occurrence of 3ADON isolates has increased, although not to the same extent as observed in Eastern Canada (Gale *et al.*, 2007; Guo *et al.*, 2008; Ward *et al.*, 2008). Population structure analysis with chemotype surveys in North America indicated that the chemotypes were from divergent populations, contradicting the previous observations of a single population in North America (Gale *et al.*, 2007; Ward *et al.*, 2008; Puri and Zhong, 2010). Furthermore, the fact that they were sympatric suggests evolutionary forces were acting differently on these populations, which may have implications for current FHB management strategies.

The high genetic diversity found in *F. graminearum* field populations in Germany was correlated with observed phenotypic diversity such as aggressiveness, DON production and fungicide sensitivity (Talas *et al.*, 2012, 2016; Talas and Mcdonald, 2015). Recent single nucleotide polymorphism analysis on whole genomes sequences of six isolates from France demonstrated a high level of polymorphism (Laurent *et al.*, 2017). Furthermore, the diversification was predominantly found in protein encoding regions, including genes involved in host adaptation (Laurent *et al.*, 2017). Such diversity could result in the selection for more aggressive isolates, undermining resistant varieties and fungicides. The emerging 3ADON population in North America poses a potential threat to the sustainability of these management tools. The 3ADON isolates were reported to be more aggressive than the 15ADON isolates, thus raising concerns regarding more severe disease outbreaks and increased DON content in grain as the population continues to change (Puri and Zhong, 2010). Recent genome sequencing in representatives of the

endemic 15ADON and introduced 3ADON population has highlighted the presence of population specific recombination hotpots with predicted functions in pathogenicity, fungicide sensitivity, and mycotoxin production (Kelly and Ward, 2018). Many of these had signatures of selective evolution, highlighting the potential impact of agronomic practices on this pathogen. The authors also noted that much of the diversity was from standing variation in the population, providing future adaptive ability in this pathogen.

3 THE IMPACT OF TRIAZOLE FUNGICIDE APPLICATION ON THE DIVERSITY OF *FUSARIUM GRAMINEARUM* FIELD ISOLATES

3.1 Abstract

Application of synthetic fungicides is part of an integrated pest management strategy (IPM) for the control of Fusarium head blight (FHB); however field efficacies have been variable. The main causal agent of FHB in Canada is *Fusarium graminearum*. This species has multiple characteristics that can drive rapid adaption to the environment. High genetic diversity has been demonstrated in field populations, supporting the species' ability to adapt to different conditions, which may be a contributing factor in the observed variation in fungicide efficacy. The response of indigenous field populations of *F. graminearum* on spring wheat to triazole fungicide application in southern Manitoba was assessed. Field trials at two farm locations with four replicated plots of untreated control and fungicide treatments at recommended and late timings were sampled for infected wheat spikes in 2017. Recovered *F. graminearum* isolates were genotyped using Amplified Fragment Length Polymorphisms (AFLPs) and the genetic response of *F. graminearum* field populations to fungicide treatment was assessed. Population structure analysis of sampled isolates from each trial site indicated the presence of two sympatric subpopulations that corresponded to previously described populations in the Upper Midwestern U.S. and Western Canada (NA1 and NA2). While there were strong associations of subpopulations and trichothecene genotype, a high number of admixtures and isolates with trichothecene genotype not predictive of subpopulation membership indicate recombination between these subpopulations. Fungicide treatment was not a significant source of genetic

variation, however, minor genetic differentiation among fungicide treatments was observed for isolates from the endemic NA1 and predominantly 15ADON producing subpopulation. The results indicate that fungicide application is not driving a shift in the *F. graminearum* population within a single growing season. However, further studies from a wider geographical area, different agro-environments including different host plants, and over multiple years would be required to fully understand the impact of triazole fungicide application on *F. graminearum* population dynamics.

3.2 Introduction

Fusarium graminearum is the predominant causal agent of Fusarium head blight (FHB) in North America (McMullen *et al.*, 2012). This disease has both direct effects on yield and indirect effects on grain quality and safety through damage to harvested kernels and mycotoxin production (Windels, 2000). Control of FHB requires an IPM approach, encompassing use of resistant varieties, fungicide application, and cultural control practices (McMullen *et al.*, 2008; Gilbert and Tekauz, 2011; Willyerd *et al.*, 2012). Efficacy of fungicide application has been shown to be highly variable which could, in part, be due to the variability in local *F. graminearum* populations (Paul *et al.*, 2008).

Fusarium graminearum populations recovered from sampled symptomatic wheat spikes can have high genotypic diversity, even within relatively small areas (Miedaner *et al.*, 2008). Zeller *et al.*, (2003) demonstrated high haplotype diversity within a 0.25m^2 area of a field. In North America, most genetic variation occurs within field populations, with little differentiation across larger geographical scales such as at state or provincial levels (Mishra *et al.*, 2004; Zeller *et al.*,

2004; Fernando *et al.*, 2006). Such high genetic diversity and large population size represents high evolutionary potential of this pathogen and thus its potential for adaptation to disease management practices, including the application of fungicides. High genetic diversity found in *F. graminearum* field populations correlated with observed phenotypic diversity in traits including aggressiveness, DON production and triazole fungicide tolerance (Talas *et al.*, 2012, 2016; Talas and Mcdonald, 2015).

Population studies have provided evidence of a change in the population dynamic of *F. graminearum* across North America that may be indicative of an emerging, more aggressive population. This has been demonstrated using molecular trichothecene chemotype profiling and population structure analysis using molecular markers (Ward *et al.*, 2008; Liang *et al.*, 2014; Kelly *et al.*, 2015). A clear shift in the chemotypes of sampled isolates from those that produce predominantly 15-acetyldeoxynivalenol (15ADON) to those that produce predominantly 3-acetyldeoxynivalenol (3ADON) trichothecenes has been demonstrated in Western and Maritime provinces of Canada and the Midwestern U.S. (Gale *et al.*, 2007; Guo *et al.*, 2008; Ward *et al.*, 2008; Kelly *et al.*, 2015). The 3ADON isolates are reported to be more aggressive than the 15ADON isolates (Puri and Zhong, 2010). This raises the concern of more severe disease outbreaks and increased DON content in grain as the population continues to change over time. Genomic analysis of North American *F. graminearum* isolates representing endemic (15ADON) and recently introduced (3ADON) populations have identified population specific gene variants predicted to be involved in a range of biotic and abiotic environment interactions (Kelly and Ward, 2018). How this variation affects the efficacy of fungicide application in the field remains to be fully investigated.

This study addresses the hypothesis that triazole fungicide application acts as a selective force on genetically diverse *F. graminearum* field populations, even within a single growing season.

3.3 Materials and Methods

3.3.1 Sampling of FHB infected spikes from 2017 field trials

Two field trials of Canada Western Red Spring (CWRS) wheat were conducted in the 2017 crop season by Holly Derksen, Manitoba Agriculture. The trials represented "natural" farm environments in that they were located on fields not previously used for research purposes. Soybean was the previous crop at both sites. The trial design consisted of three treatments~: untreated (no fungicide applied), triazole fungicide application (Prosaro®, Bayer, Leverkusen, Germany) at recommended rate and timing (early flowering), and late fungicide application at the recommended rate (seven days after recommended timing). The untreated plots were 50 m x 50 m at the edge of the field. The recommended timing treatment plots extended from the end of the untreated plot to the end of the field. The start of the late timing treatment plots were adjacent to the untreated plots and extended the full length of the field. Site locations, variety and fungicide treatment details are provided in Table 3-1.

Table 3-1 2017 Field trial site locations, wheat varieties and fungicides used

Trial name	Field co-ordinates	Variety (name/class*/FHB resistance rating**)	Fungicide (name, active ingredients)
St. Jean Baptiste	49°17'37.6"N 97°17'53.5"W	AAC Elie/CWRS/Intermediate resistance	Prosaro®, Prothioconazole and Tebuconazole
Brunkild	49°36'22.0"N 97°30'57.6"W	AAC Brandon/CWRS/Moderate resistance	Prosaro®, Prothioconazole and Tebuconazole

*CWRS- Canada Western Red Spring
**Seed Manitoba, 2017

Weather data were collected from Manitoba Agriculture weather stations from seeding to harvest (Brunkild May 5th to August 31st, St. Jean Baptiste April 29th to August 22nd). The nearest weather station to the Brunkild site was located 1 km south of the trial site (49°35'27.2"N 97°31'04.4"W). The nearest weather station to the St. Jean Baptiste site was located 8 km north of the trial site (49°21'37.8"N 97°19'17.4"W). Daily average temperatures (°C) and precipitation (mm) were recorded.

Field plots were assessed for FHB disease levels twenty five days (+/- one day) after the fungicide application at the recommended timing by Manitoba Agriculture. Thirty randomly chosen spikes per plot were assessed for FHB incidence (calculated as the percentage of spikes with FHB disease symptoms in each plot) and severity (calculated as the percentage of spikelets with disease symptoms, averaged across all spikes with disease symptoms per plot). FHB index for each plot was calculated using the following equation:

$$FHB\ index = \frac{incidence\ x\ severity}{100}$$

Plots were harvested by the grower and grain samples from each plot were sent for grain grading by Canadian Grain Commission inspectors following procedures outlined in the Official Grain Grading Guide (Canadian Grain Commission, 2019). Deoxynivalenol (DON) content analysis was performed by the Canadian Grain Commission, using the Neogen® Accuscan Gold® enzyme-linked immunosorbent assay (ELISA) (Neogen, MI, US). Grain samples were analysed for individual plots from recommended and late fungicide treatments. Grain samples from the untreated plots were combined into one pooled sample prior to grain analyses.

Just prior to full maturity, twenty spikes showing FHB disease symptoms were collected from each plot at random (*i.e.* a total 240 spikes per trial site). Spikes were air dried at room temperature for one month prior to storing at -30°C.

3.3.2 *Fusarium* species isolation from wheat spikes

A pure single spore culture was obtained from each spike. Five to six kernels were manually removed from the spike and surface sterilised by soaking in 0.3% sodium hypochlorite for one minute. Kernels were placed on sterile filter paper and allowed to air-dry in a biological safety cabinet for one hour and then plated onto 9 cm petri dishes containing 0.5X potato dextrose agar (PDA) plus 100 ppm streptomycin (See Appendix 7.1). Plates were incubated at 21°C under continuous ultraviolet and fluorescent white light until species characteristics were fully developed (seven to ten days). *Fusarium graminearum* was identified visually based on colony morphology, red mycelial pigmentation on the reverse side of the culture plate and the

production of orange sporodochia. Further assessment of conidia morphology under a light microscope was performed when culture characteristics were uncertain. Under a dissecting microscope, a single sporodochium was selected from a single phenotypically unique colony using a sterile needle. The sporodochium was mixed in a 1.5 ml micro-centrifuge tube containing 500 µl of autoclaved acidified water (two drops of 25% lactic acid / 10 ml water) to suspend macroconidia. The macroconidia suspension was spread onto a 9 cm petri dish containing PDA and incubated as described above for 16 to 20 hours to allow for germination of conidia. Using a light microscope, a square of agar encompassing a single germinated conidium was carefully cut from the agar plate and transferred to a 6 cm petri dish containing synthetic nutrient agar (SNA) (See Appendix 7.1) and a 1 cm^2 piece of sterile Whatman no. 3 filter paper (Whatman plc, GE Healthcare, IL, US). Plates were incubated as previously described until sporodochia could be seen with the naked eye (10-14 days).

Fungal isolates were placed into long-term and short-term storage using single spore cultures from the SNA culture plates. For short term storage, five agar plugs with sporodochia were taken from cultures on SNA and placed in a 2 ml cryogenic vial containing 1 ml of sterile water and stored at 4 °C. For long term storage, an additional five agar plugs were taken from cultures on SNA and placed in a 2 ml cryogenic vial containing 1 ml of sterile 10% glycerol and then stored at -145 °C.

3.3.3 Fungal DNA extraction

Fusarium graminearum DNA was extracted from mycelium and spores harvested from seven to ten day PDA plate cultures grown under the same conditions as detailed in Section 3.3.2. Spin

column DNA extraction was performed using a NucleoSpin® Plant II kit (Macherey-Nagel, Düren, Germany) following the manufacturers protocol based on a cetyl trimethylammonium bromide lysis buffer method. Extracted DNA was eluted in 100 µl of elution buffer. The DNA was concentrated in a DNA Speedvac (Savant Instruments, Farmingdale, NY), re-suspended to 30 µl with molecular grade water and stored at -30°C. DNA from each isolate was quantified using the Quant-iT™ Qubit™ dsDNA HS Assay Kit (Invitrogen Life Technologies, Carlsbad, CA).

3.3.4 Species confirmation and trichothecene genotyping

Fusarium graminearum species identification and trichothecene genotype were confirmed using the Wafergen Smartchip real-time PCR system (WaferGen Biosystems, Fremont, CA). A panel of six primer pairs was used to confirm *F. graminearum* species and 15ADON, 3ADON, NIV or NX-2 genotype (Table 3-2). All primer sets excluding NX-2 were from previously validated studies. The primers for NX-2 were developed at Agriculture and Agri-Food Canada, Ottawa (Schneiderman, unpublished). The primers were designed to amplify a 65 bp product from the *Tri1* allele specific to NX-2 isolates. Amplification was conducted in a 100 nl reaction in a WaferGen SmartChip at a final concentration of 1 X LightCycler 480 SYBR Green I Master Mix (Roche Inc., USA), 0.05-1.4 ng/µl DNA template and 250 nM of each forward and reverse primer, dispensed using the WaferGen SmartChip Multi-sample Nanodispenser. Thermal cycling was performed using a Wafergen SmartCycler as follows: initial denaturation at 95 °C for 173s, 40 cycles of denaturation at 95 °C for 34s, annealing at 60 °C for 74s. Specificity of amplification was accessed with a melt curve analysis from 60 °C to 97 °C at a ramp rate of 0.4 °C / step. The results of the real time polymerase chain reaction (PCR) were analysed using

SmartChip qPCR software (V2.7.0.1). Wells with expected peaks that deviated more than 1 °C above or below expected primer melting temperature (Tm) were disregarded. A cycle threshold (Ct) of 31 was used as the limit of detection.

To examine the treatment differences in the ratio of 15ADON and 3ADON trichothecene genotypes for each location, Chi-squared tests with $P < 0.05$ were considered as significant (performed in statistical software package R version 3.0.2).

Table 3-2 Specific PCR primers used for confirmation of *Fusarium graminearum* identification and trichothecene genotype (3ADON, 15ADON, NIV and NX-2).

Target	Primer Name	Product size (bp)	Sequence (5′–3′)	Reference
Fusarium graminearum (RAPD fragment)	Fg16N-F	280	ACAGATGACAAGATT CAGGCACA	Nicholson *et al.*, 1998
	Fg16N-R		TTCTTTGACATCTGTT CAACCCA	
Fusarium graminearum (elongation factor 1 α)	FgramB379 fwd	~60	CCATTCCCTGGGCGCT	Nicolaisen *et al.*, 2009
	FgramB411 rev		CCTATTGACAGGTGGT TAGTGACTGG	
15ADON (*TRI12*)	15ADONfwd	60	GTTTCGATATTCATTG GAAAGCTAC	Nielsen *et al.*, 2012
	15ADONrev		CAAATAAGTATCGTCT GAAATTGGAAA	
3ADON (*TRI12*)	3ADONfwd	57	AACATGATCGGTGAG GTATCGA	Nielsen *et al.*, 2012
	3ADONrev		CCATGGCGCTGGGAG TT	
NIV (*TRI12*)	NIVfwd	77	GCCCATATTCGCGACA ATGT	Nielsen *et al.*, 2012
	NIVrev		GGCGAACTGATGAGT AACAAAACC	
NX-2 (*TRI1*)	NX1242tri1-fwd	65	TCGATGTTAATTGTTT TTGTGTA	Schneiderman., unpublished
	NX1243tri1-rev		AGCCAGCTGGGTTCTT G	

3.3.5 Generation of amplified fragment length polymorphism markers

Amplified fragment length polymorphism markers (AFLPs) were generated following standard protocols with some modifications as described (Vos *et al.*, 1995; Leslie and Summerell, 2006). Genomic DNA (18-45 ng) was incubated for 2 h at 37 °C with 1 U of *Mse1* and 1 U *EcoR1* in

12.5 µl 1 NEB Cutsmart Buffer (New England Biolabs, MA, US). The mixture was cooled to 20 °C and added to 12.5 µl of a solution containing 5 pMol *EcoRI* adapters, 50 pMol *Mse1* adapters, 20 U T4 DNA-ligase in 1x NEB Ligase reaction buffer (New England Biolabs, MA, US) and incubated for 2 h at 20 °C. Adapters were obtained as pre-synthesised oligo duplexes (Integrated DNA Technologies, IL, US). After ligation the reaction mixture was incubated for 20 min at 65 °C and a 10 µl aliquot diluted to 100 µl in 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA and stored at -30°C.

AFLP template amplification was performed using a pre-amplification step followed by a selective amplification. The 25 µl pre-amplification reactions contained 2.5 µl diluted digestion/ligation mixtures, 0.2 µM of each *EcoRI* pre-amplification primer (complementary sequence to the *EcoRI* adapter) and *MseI* pre-amplification primer (complementary sequence to the *MseI* adapter), 0.2 mM dNTPs, 1x buffer, 2 mM MgCl2, and 0.5 U of *Taq* DNA polymerase in molecular grade water. The pre-amplification cycle was performed with a denaturation for 5 min at 94 °C followed by 20 cycles of 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s with a final extension step of 5 min at 72 °C. A 2.5 µl aliquot of the pre-amplification reaction was diluted to 100 µl in 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA, and stored at -30°C. Four sets of primer combinations: *EcoR1*+CC/*Mse1*+CG, *EcoR1*+TG/*Mse1*+CG, *EcoR1*+AA/*Mse1*+CG, and *EcoR1*+AA/*Mse1*+CC) were used in the selective amplification, with the *EcoR1*+NN primers labelled with either the fluorescent dye IRD 700 or IRD 800 (LI-COR, NE, US). The 10.5 µl reactions contained 2 µl of diluted pre-amplification reaction, 0.06 µM of *EcoRI*+NN primer, 0.2 µM *MseI*+NN, 0.2 mM dNTPs, 1x buffer, 2 mM MgCl2, and 0.5 U of *Taq* DNA polymerase in molecular grade water. The selective amplifications were performed with a touch down

programme for 13 cycles: 94 °C for 30 sec, 65 °C for 30 sec, 72 °C for 60 sec with a 0.7 °C decrease of annealing temperature each cycle, followed by 23 cycles of amplification at 94 °C for 30 sec, 56 °C for 30 sec, and 72 °C for 60 sec. Reactions were stored at -30°C.

Two isolates with different haplotypes were used as positive controls along with a negative non-template DNA control for all steps from restriction digestion onwards in all independent sets of reactions (*i.e.* reactions set up from the same master mix and amplified at the same time). AFLP reactions were performed on five DNA extraction controls (no fungal DNA) to assess for potential background contamination in all DNA extraction kit batches.

3.3.6 Gel Electrophoresis

Two selective amplification reactions with different fluorescent dye wavelengths were combined 1:1 to a volume of 10 µl and mixed with 5 µl of formamide loading dye, heated to 95°C for 5 min, immediately chilled on ice, and loaded (0.4 µl) on a polyacrylamide gel in the LI-COR 4300 DNA analyser system (LI-COR). The gel was prepared by using 28 ml of 6.5% LI-COR KB^{Plus} (LI-COR), 20 µl of tetramethylethylenediamine and 210 µl freshly prepared 10% ammonium persulfate. A 50 – 1500 bp two-colour (IRD 700 / IRD 800) DNA sizing standard (LI-COR) was run on the first and last lane of the gels.

Amplified fragment length polymorphism fragments of 70 to 600 bp were scored using SAGA^{MX} AFLP^{TM} software (LI-COR). Fragments that were absent in at least one isolate, but present in other isolates were considered to be polymorphic. Fragments were scored in a binary present (1) / absent (0) format. Bands with the same mobility in different individuals were assumed to be

homologous and represented the same allele. Error rate was assessed by repeating 10% of a randomly chosen set of isolates from independent DNA extractions for each primer pair. Error rate was calculated as the percentage of the total number of mismatches (band presence vs. band absence) to the number of replicated isolates. Individual loci with an error rate above 5% were not included in the final data analysis.

3.3.7 AFLP data analysis

To assess whether the number of polymorphic markers (74 for the Brunkild dataset, 75 for the St. Jean Baptiste dataset) had the power to discriminate between unique multilocus genotypes (MLGs), genotype accumulation curves were generated using the R package *poppr* (Kamvar *et al.*, 2015). Clone correction was performed at the level of individual field study sites and fungicide treatments using the R package *poppr*. Pairwise PhiPT (Φpt), an equivalent of the fixation index (Fst) for haploid data, was estimated between sites using GenAlEx 6.2 (Peakall and Smouse, 2006) with 1000 permutations to test for statistical significance.

The Bayesian based model clustering method in STRUCTURE 2.3.4 software (Pritchard *et al.*, 2000) was used to test for population subdivision and to examine admixture among populations within each field study site. All *F. graminearum* isolates were assigned to *K* populations with *K* ranging from 1 to 7 based on their AFLP genotype using the admixture model and independent allele frequency model. Ten replicate runs were performed for each value of *K* with all runs performed with a 25,000 burnin period and 100,000 Monte Carlo Markov Chain repeats after burnin. The Evanno *et al.*, (2005) method was used to select the optimal model that maximised the rate of change in the log likelihood values, ΔK. The proportion of membership (*q*) in each of

the *K* clusters was determined for each isolate. Genalex 6.2 was used to calculate unbiased gene diversity (*H*) for subpopulations defined by the STRUCTURE analysis according to Nei, (1973).

AMOVA was performed using the R package *poppr* to partition the molecular variance at each trial site into three hierarchical levels: within subpopulations inferred from STRUCTURE analysis, among the three field fungicide treatments described in Section 3.3.1 within subpopulations, and individuals within field fungicide treatment within subpopulations (Excoffier *et al.*, 1992). The statistical significance of estimated Φ-statistics and partitioned molecular variance at each level was tested using 1000 nonparametric permutations according to Excoffier *et al.*, (1992). To further assess genetic variation between STRUCTURE assigned subpopulations and fungicide treatment, a discriminant analysis of principal components (DAPC) was performed in the R package *adegenet* (Jombart *et al.*, 2010). DAPC reduces the number of variables first into principle components, and then uses a subset of these to minimise within group variation and maximise among group variation and would better detect small differences between the defined groups (Jombart *et al.*, 2010).

3.4 Results

3.4.1 Field trial data

The visual FHB index assessments showed that across both the St. Jean Baptiste and Brunkild sites the disease levels were relatively low in all treatments, including the untreated plots (Figure 3-1). St. Jean Baptiste had a lower average FHB index than Brunkild in the untreated plots (0.70

vs. 2.54 respectively). Fungicide application at both the recommended and late timing reduced FHB index compared to the untreated in both trials.

Grain grading data (grade, fusarium damage (FUS DMG)) and DON content data for harvest samples are presented in Table 3-3. Grain samples from all plots with fungicide treatment (at recommended and late timing) were designated No. 1 grade. The pooled untreated samples from St. Jean Baptiste and Brunkild were designated feed grade and No. 2 grade, respectively. FUS DMG is measured as a percent by mass of kernels with white or pinkish mould or fibrous growth (Canadian Grain Commission, 2019). FUS DMG ranged from not present to very low in all samples (maximum 0.1% average in the St. Jean Baptiste recommend fungicide timing samples). FUS DMG could not be quantified in the St. Jean Baptiste untreated sample due to the presence of mouldy kernels, which could be due to *Fusarium* and/or other fungal species. DON content was highest in the St. Jean Baptiste untreated grain sample with 1.2 ppm detected. This suggests that the mouldy kernels were due at least partly to *Fusarium* infection. All other samples had DON at, or below, the limit of quantification for the ELISA assay.

Weather data from the nearby weather stations show that both sites had similar temperatures (Brunkild Figure 3-2, St. Jean Baptiste Figure 3-3). Over the month of July, when wheat would be in flower, and therefore most susceptible to FHB, precipitation was higher near the St. Jean Baptiste site than the Brunkild site (total precipitation 54.25 mm *vs.* 37.36 mm).

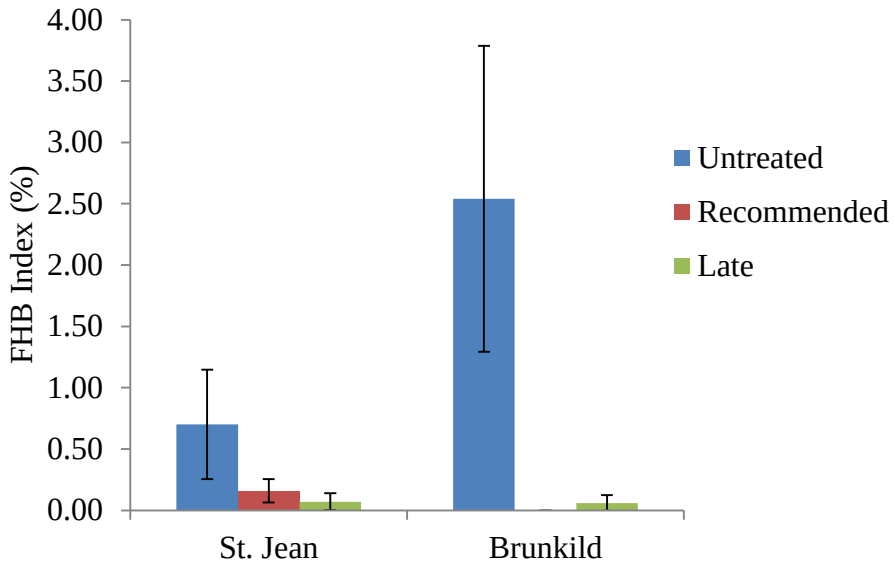

Figure 3-1 FHB index across St. Jean Baptiste and Brunkild field trial fungicide treatments averaged across four replicate plots. Error bars represent +/- Standard Error (SE) calculated from the FHB index based on thirty spikes from each of four replicate plots.

Table 3-3 Canadian Grain Commission grain grading (overall grade and *Fusarium* damage (FUS DMG)) and DON content data for the St. Jean Baptiste and Brunkild field trial harvested grain samples. Untreated samples from the four replicate plots were pooled prior to analyses. All other data are the mean of grain samples from four replicate plots that were analysed individually.

Field location	**Fungicide Application Timing**	**Grain Grade**	**FUS DMG**	**DON (ppm)**
St. Jean Baptiste	Untreated	Feed	Mouldy	1.2
	Recommended	No. 1	0.10	<0.3
	Late	No. 1	0.05	<0.3
Brunkild	Untreated	No. 2	0.0	<0.3
	Recommended	No. 1	0.0	<0.3
	Late	No. 1	0.0	<0.3

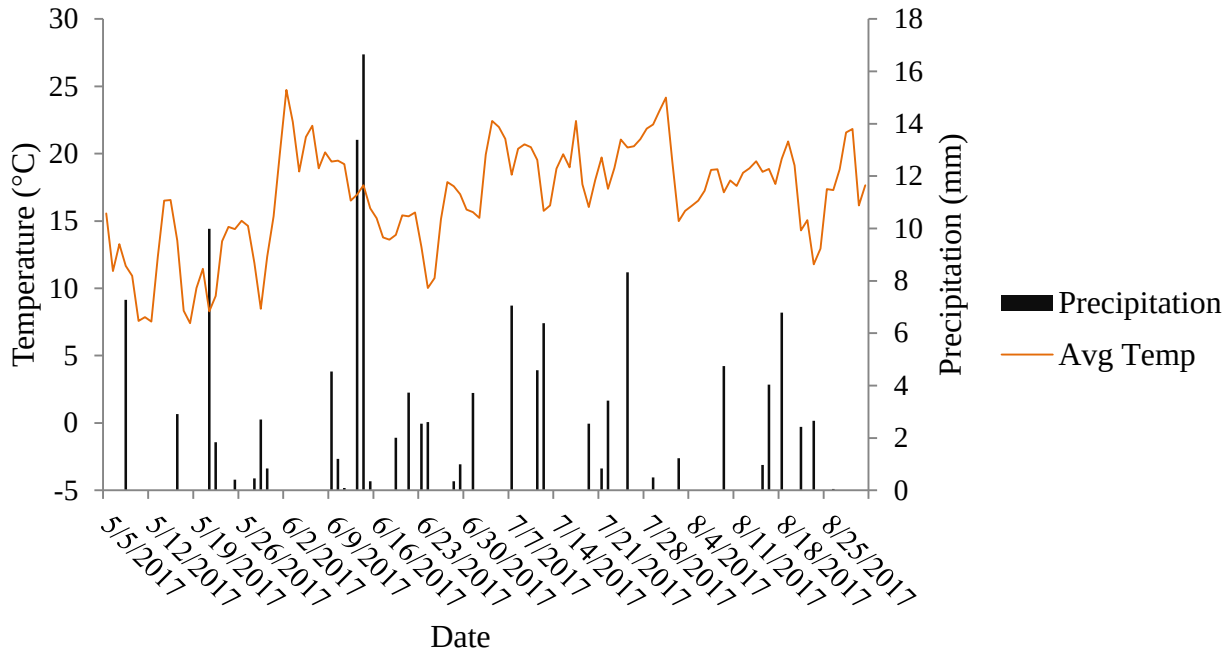

Figure 3-2 Daily precipitation and average temperature data for the nearest weather station to the Brunkild trial site from May 5th 2017 to August 31st 2017.

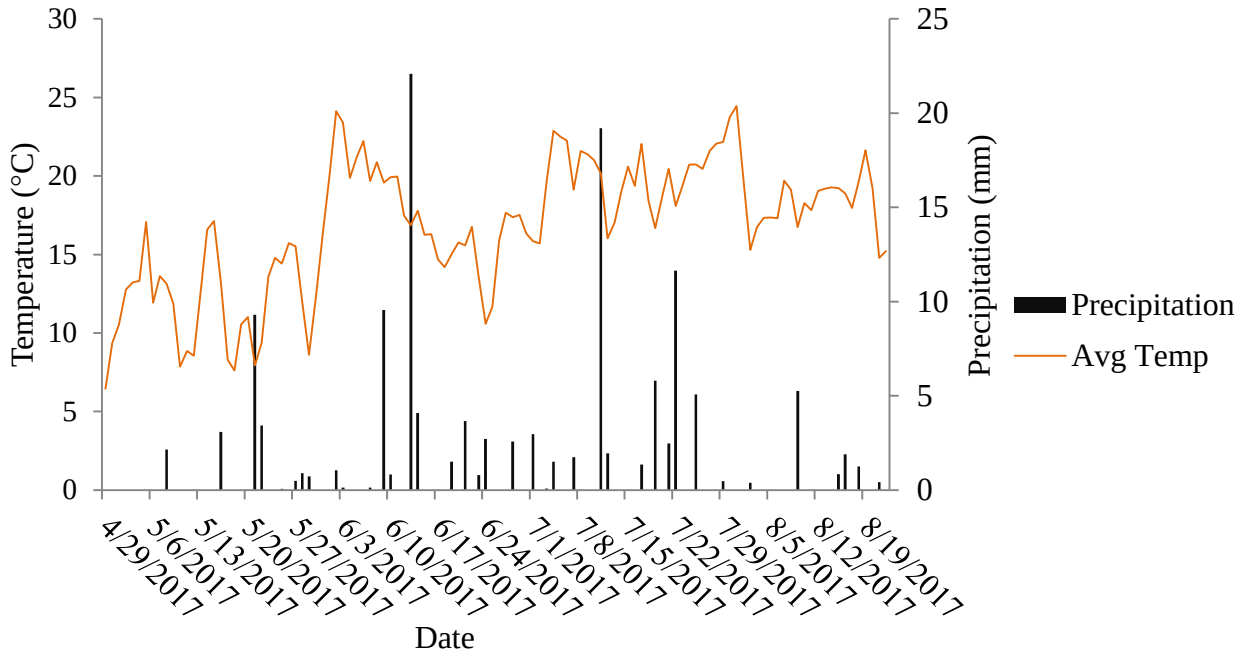

Figure 3-3 Daily precipitation and average temperature data for the nearest weather station to the St. Jean Baptiste trial site from April 29th 2017 to August 22nd 2017.

3.4.2 Fungal isolation and characterization

A total of 238 single spore cultures were isolated from the Brunkild wheat spike samples. Of these isolates, 231 were confirmed through species specific PCR analysis as *F. graminearum*. Three spikes each produced two phenotypically distinct *F. graminearum* colonies from a single spike and so single spore isolates were obtained from each colony. All other single spore isolates came from a single spike. Re-isolations from the spikes sampled from the St. Jean Baptiste site resulted in 243 single spore isolates, of which 231 were confirmed to be *F. graminearum*. Five spikes each produced two phenotypically distinct *F. graminearum* colonies from a single spike and so single spore isolates were obtained from each colony. All other single spore isolates came from a single spike. The number of isolates obtained for each trial plot for both sites ranged from 18 to 21.

The overall frequency of trichothecene genotypes in the Brunkild trial was 27.3% 15ADON and 72.7% 3ADON. There were no NIV or NX-2 genotypes. The frequency ranged from 26.0% 15ADON and 74.0% 3ADON in the no fungicide treatment (untreated) to 30.3% 15ADON and 69.7% 3ADON in the late fungicide application treatment (Figure 3-4). There was no significant difference in the frequency of trichothecene genotypes across treatments (X^2 = 0.51, P>0.05). The overall frequency of trichothecene genotypes in the St. Jean Baptiste trial was 30.3% 15ADON and 69.7% 3ADON; there were no NIV or NX-2 genotypes. The frequency ranged from 35.6% 15ADON and 69.7% 3ADON in the no fungicide treatment (untreated) to 26.0% 15ADON and 75.0% 3ADON in the recommended fungicide application treatment (Figure 3-5). There was no significant difference in the frequency of trichothecene genotypes across treatments (X^2 = 1.68, P>0.05).

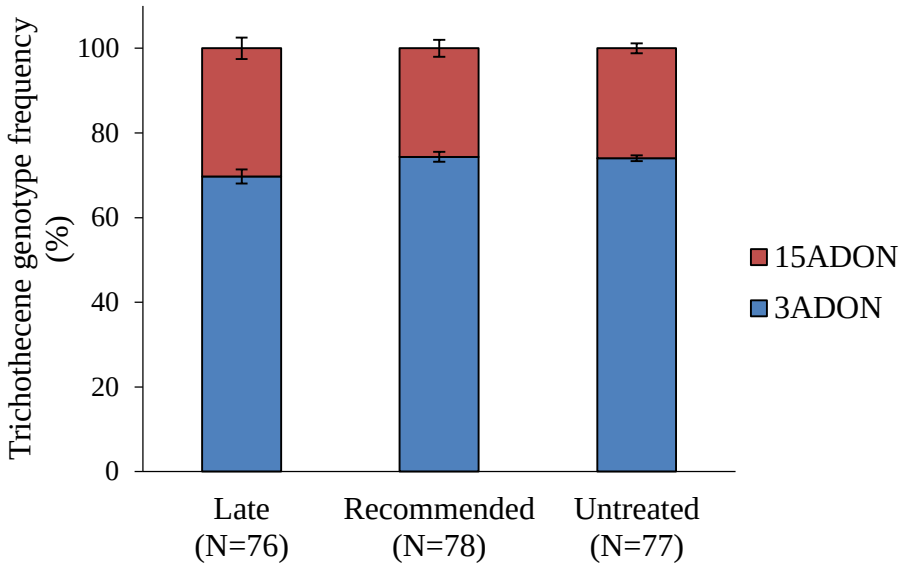

Figure 3-4 The frequency of trichothecene genotypes across field treatments from 231 *F. graminearum* isolates isolated from wheat spikes sampled from the Brunkild trial site. Error bars represent +/- standard error calculated from the frequencies in each replicate field plot

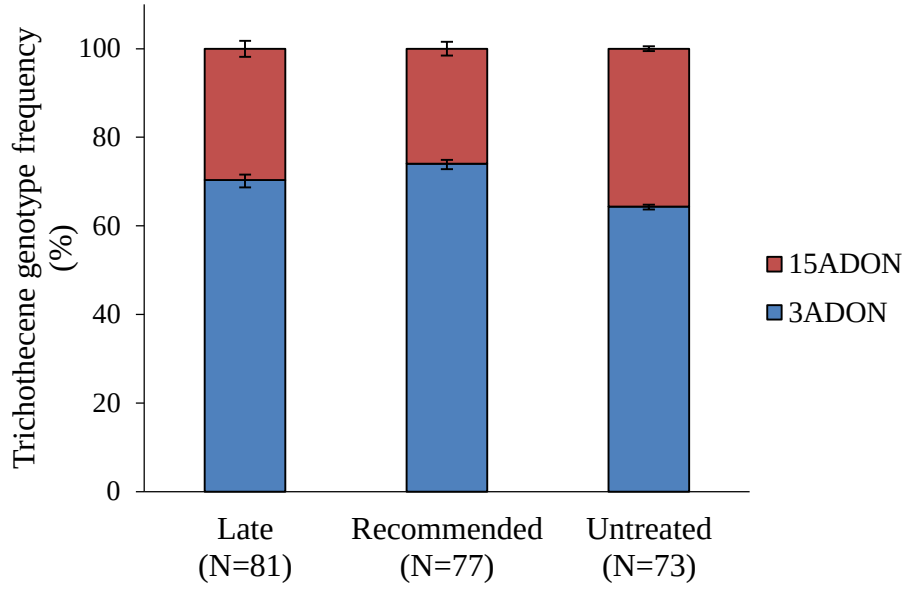

Figure 3-5 The frequency of trichothecene genotypes across field treatments from 231 *F. graminearum* isolates isolated from wheat spikes sampled from the St. Jean Baptiste trial site. Error bars represent +/- standard error calculated from the frequencies in each replicate field plot.

3.4.3 AFLP data quality and multilocus genotypes

In total, 75 polymorphic AFLP markers were successfully scored across 229 of the 231 Brunkild *F. graminearum* isolates and 224 of the 231 St. Jean Baptiste *F. graminearum* isolates. One marker was polymorphic in the St. Jean Baptiste isolates only, and was therefore not included in further analysis on the Brunkild dataset unless it was combined with the St. Jean Baptiste dataset. The overall AFLP scoring error rate based on the randomly chosen independent DNA extractions was 2.5%.

Genotype accumulation curves for both sites showed that a plateau for the number of MLGs was reached with the total number of polymorphic loci (75 for St. Jean Baptiste, 74 for Brunkild) with decreasing variance, indicating that a sufficient number of markers was used to discriminate individual genotypes (Figure 3-6 and Figure 3-7). In total, 227 MLGs were identified in the Brunkild dataset and 221 MLGs were identified in the St. Jean Baptiste dataset, demonstrating high haplotype diversity in both sites. No individuals of the same MLG were found within a single field trial plot, and only two individuals with the same MLG were from the same field treatment. When datasets were combined, 437 MLGs were identified across the full 453 individuals, demonstrating more individuals with the same MLG were found across sites than within sites. Of all MLGs with multiple individuals either across or within individual sites (n=30), 28 were 3ADON producers and two were 15ADON producers. Based on the AFLP loci, the two 15ADON producers were the same genotype as a 3ADON producer. Φpt between the sites was 0.003 (P=0.029) demonstrating that the St. Jean Baptiste and Brunkild isolates had significant, but low genetic differentiation.

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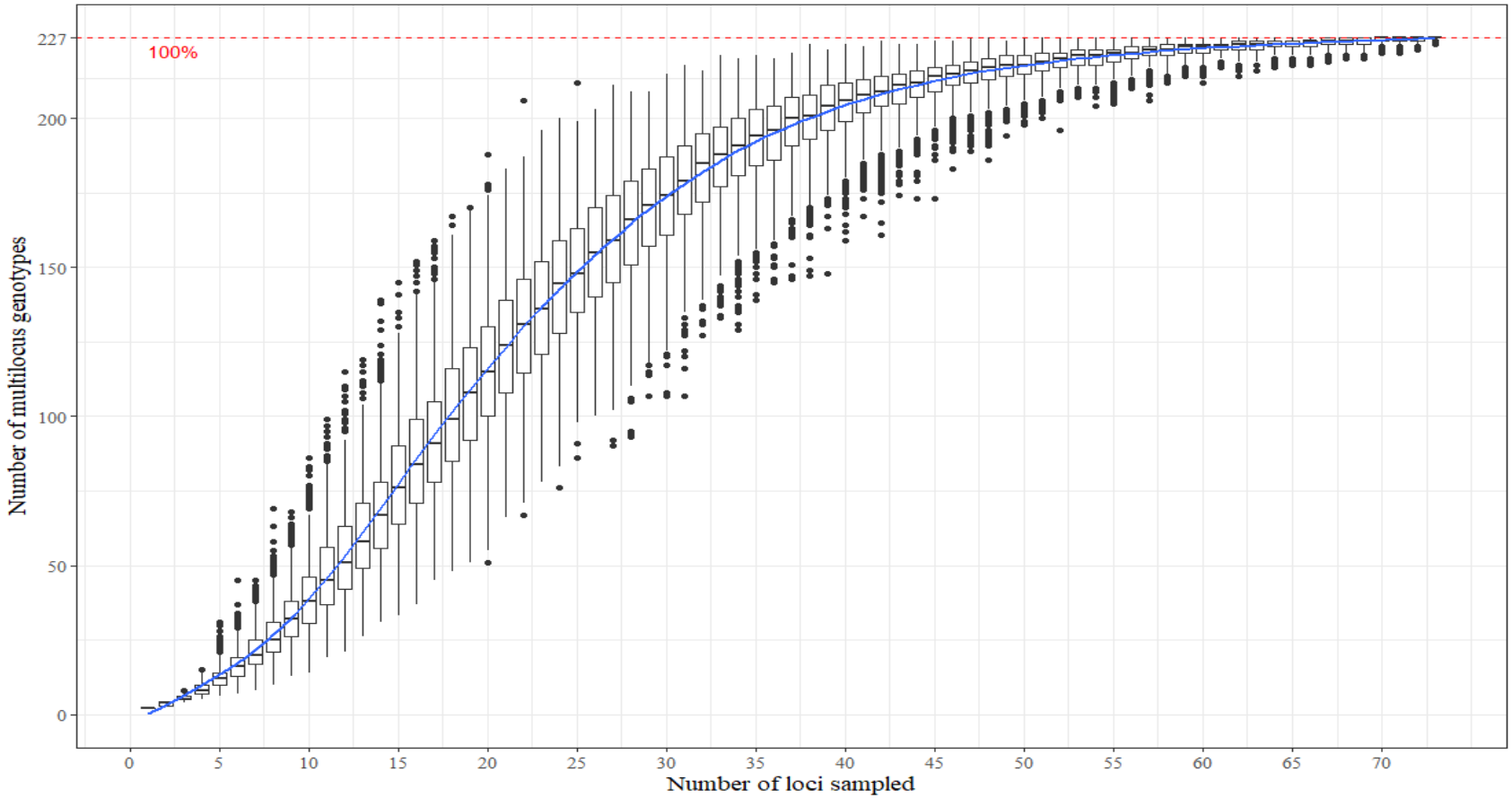

Figure 3-6 Genotype accumulation curve for 229 isolates genotyped from the Brunkild site. Boxplots show the distribution of the number of multilocus genotypes (MLGs) estimated from 1000 random samples of *n* (1-73) loci (AFLP markers). Boxes indicate the median (solid horizontal line), 25th and 75th percentiles respectively. Vertical lines show the minimum and maximum and black dots refer to outlying data points. 100% of the number of MLGs genotypes identified in each population is indicated by the dashed red line.

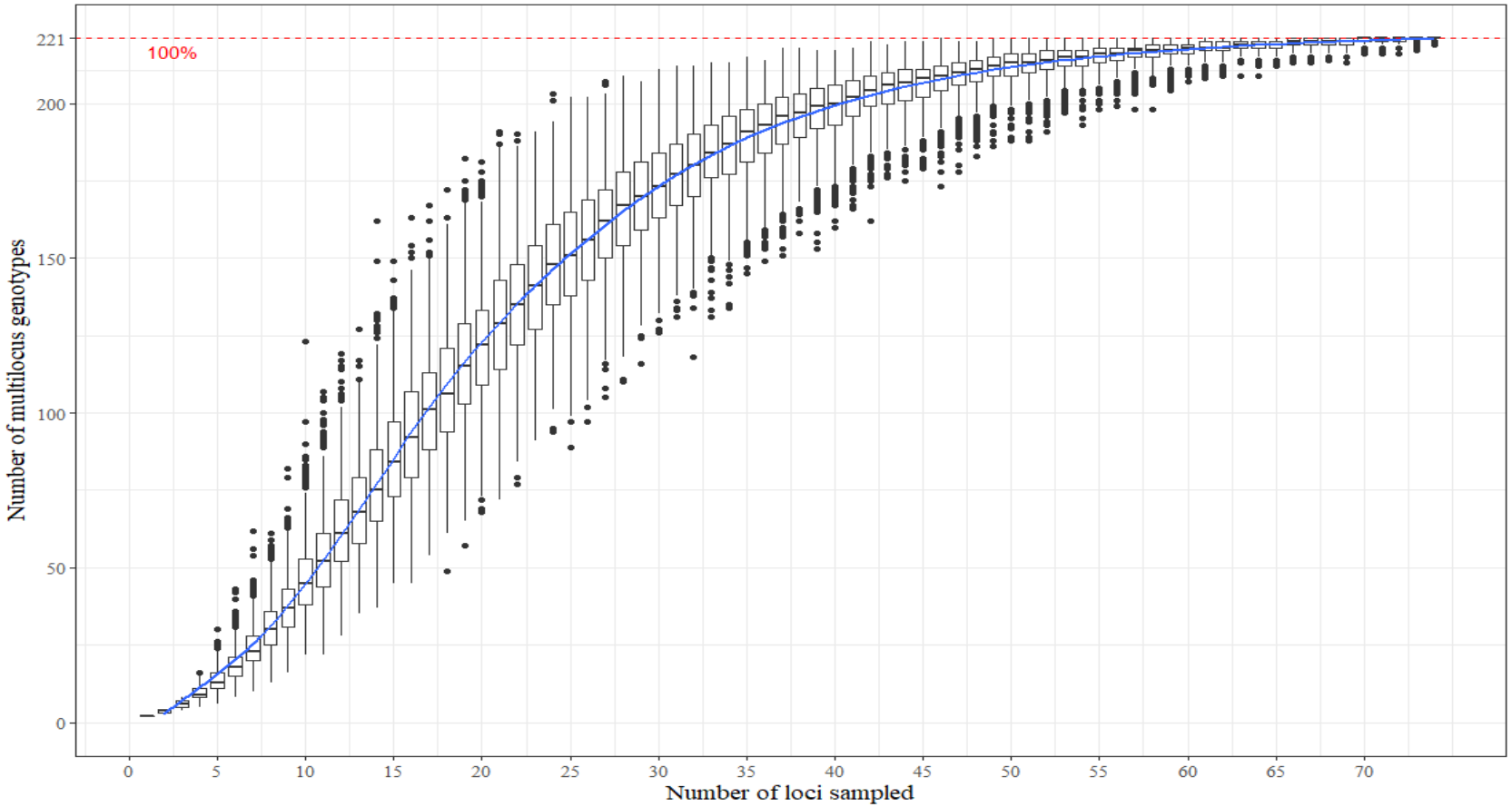

Figure 3-7 Genotype accumulation curves for 224 isolates from the St. Jean Baptiste site. Boxplots show the distribution of the number of multilocus genotypes (MLGs) estimated from 1000 random samples of n (1-74) loci (AFLP markers). Boxes indicate the median (solid horizontal line), 25th and 75th percentiles respectively. Vertical lines show the minimum and maximum and black dots refer to outlying data points. 100% of the number of MLGs identified in each population is indicated by the dashed red line.

3.4.4 Population structure within study sites

Two genetic clusters (*K*=2) were determined to capture the major genetic structure within the Brunkild and St. Jean Baptiste datasets, based on the highest rate of change in log likelihood (Δ*K*) (Figure 3-8). Clusters were described as NA1 and NA2 representing the majority of 15ADON and 3ADON trichothecene genotypes, respectively (Figure 3-9). This is in accordance with previously described *F. graminearum* populations in North America (Kelly *et al.*, 2015; Liang *et al.*, 2015). The NA1 subpopulation was dominated by isolates with 15ADON genotypes (98% 15ADON Brunkild, 92% St. Jean Baptiste), whereas the NA2 subpopulation was dominated by isolates with 3ADON genotypes (94% 3ADON Brunkild, 91% St. Jean Baptiste).

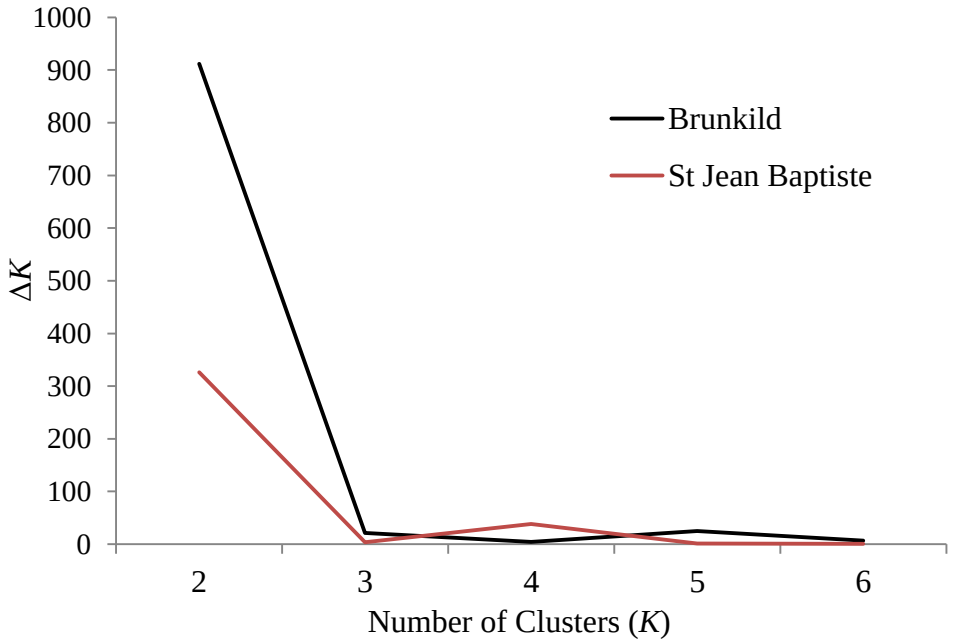

Figure 3-8 Delta *K* for Brunkild and St. Jean Baptiste datasets based on the method by Evanno et al., (2005).

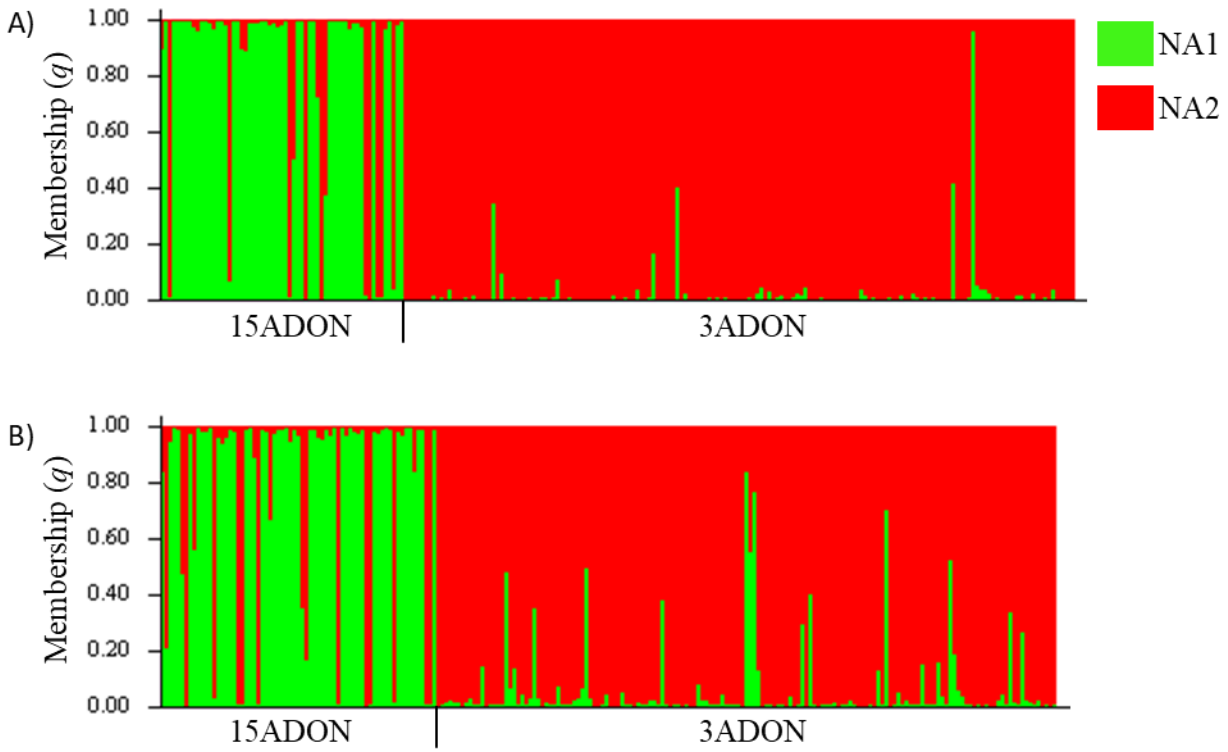

Figure 3-9 Assignment of *F. graminearum* isolates from the Brunkild site (A) and St. Jean Baptiste site (B) based on AFLP data using STRUCTURE software. Individual isolates are represented by a distinct vertical line coloured to represent the estimated proportion of the isolate's genome derived from each subpopulation (NA1 green, NA2 red). Isolates were grouped by trichothecene genotype.

The majority of isolates were assigned with high membership probability ($q \geq 0.9$) to a subpopulation that corresponded to the predominant trichothecene chemotype (*i.e.* 15ADON to NA1 and 3ADON to NA2) (90.4% Brunkild, 82.6% St. Jean Baptiste, Figure 3-9). Admixtures is a term previously used to describe *F. graminearum* isolates that A) have a low membership probability to any subpopulation, or B) have a high membership to a subpopulation, but have a trichothecene genotype contrary to subpopulation assignment (Kelly *et al.*, 2015; Liang *et al.*,

2015). In this study, admixtures were defined as both 15ADON and 3ADON isolates with a low probability of assignment to either the NA1 subpopulation or NA2 subpopulation ($0.5 < q < 0.9$) and isolates with a high probability of assignment ($q \geq 0.9$) to a subpopulation not predictive of trichothecene genotype (*i.e.* assignment to NA1 with a 3ADON genotype or assignment to NA2 with a 15ADON genotype). Three isolates from Brunkild and six isolates from St. Jean Baptiste had membership probabilities (q) close to 0.5 for each subpopulation, thus making subpopulation assignment uncertain. However, all but one isolate (SJ3LS10) were consistently assigned to either subpopulation in the ten repeated runs of the model in STRUCTURE and were therefore assigned to a single subpopulation (data not shown).

In total, admixtures represented 9.6 % and 17.9 % of all isolates in the Brunkild and St. Jean Baptiste datasets respectively (Table 3-4). A summary of the q values and trichothecene genotype for admixture isolates is provided in Table 3-5. Isolates with a low probability of membership to either NA1 or NA2 ($0.5 < q < 0.9$) represented 4.8% of all isolates from Brunkild and 12.9% of all isolates from St. Jean Baptiste. Isolates assigned to NA1 or NA2 ($q > 0.5$), but with a trichothecene genotype atypical of the subpopulation, represented 5% of all isolates from Brunkild and 8.9% of all isolates from St. Jean Baptiste. Of the twenty-two admixture isolates from the Brunkild trial site, five had a 15ADON trichothecene genotype and were assigned to the NA1 population with q values ranging from 0.503 to 0.895, eleven had a 15ADON trichothecene genotype and were assigned to the NA2 population with q values ranging from 0.63 to 0.996, five had a 3ADON trichothecene genotype and were assigned to the NA2 population with q values ranging from 0.584 to 0.893, and one had a 3ADON trichothecene genotype and was assigned to the NA1 population with a q value of 0.957 (Table 3-5). Of the forty admixture

isolates from the St. Jean Baptiste trial site, five had a 15ADON trichothecene genotype and were assigned to the NA1 population with *q* values ranging from 0.556 to 0.881, fifteen had a 15ADON trichothecene genotype and were assigned to the NA2 population with *q* values ranging from 0.661 to 0.993, fourteen had a 3ADON trichothecene genotype and were assigned to the NA2 population with *q* values ranging from 0.502 to 0.870, and five had a 3ADON trichothecene genotype and were assigned to the NA1 population with a *q* values ranging from 0.523 to 0.840. As previously discussed, isolate SJ3LS10 could not be assigned to either subpopulation and had a 3ADON trichothecene genotype (Table 3-5).

Of the admixtures with a trichothecene genotype not predictive of subpopulation with $q > 0.5$, 91.7% and 75% were 15ADON with NA2 genetic background for Brunkild and St. Jean Baptiste, respectively (Table 3-5). These isolates can also be described as recombinants and represent 18% of all 15ADON isolates and 0.6% of all 3ADON isolates from Brunkild and 16% of all 15ADON isolates and 6% of all 3ADON isolates from St. Jean Baptiste. The majority of these recombinants had a high membership probability ($q \geq 0.9$) to either subpopulation. Just one recombinant isolate (BR4RS9*)* from Brunkild had low membership probability to a subpopulation and was 15ADON genotype assigned to the NA2 subpopulation ($q = 0.630$). Of all recombinants from St Jean Baptiste with a low probability of subpopulation assignment, four isolates had a 15ADON genotype assigned to NA2 with *q* values ranging from 0.661 to 0.861 and five isolates with 3ADON genotype were assigned to NA1 with *q* values ranging from 0.523 to 0.840.

Table 3-4 Sample size (N), unbiased gene diversity (*H*), and the admixture proportion of isolates from the Brunkild and St. Jean Baptiste AFLP datasets of *Fusarium graminearum* isolates.

Dataset[a]	**N for genetic analysis (%NA1, %NA2)**	***H* (SE)**[b]		**Admixture proportion**[c]
		NA1	**NA2**	
Brunkild	299 (22,78)	0.169 (0.02)	0.164 (0.02)	9.6 %
Brunkild excluding admixtures	207 (21.7, 78.3)	0.161 (0.02)	0.159 (0.02)	-
St. Jean Baptiste	224 (26, 74)	0.197 (0.02)	0.165 (0.02)	17.9 %
St. Jean Baptiste excluding admixtures	184 (26.6, 73.4)	0.178 (0.02)	0.150 (0.02)	-

[a] Isolates with a low probability of assignment to subpopulation ($0.5 < q < 0.9$) were removed prior to analysis for datasets excluding admixtures.

[b] Nei's Unbiased gene diversity. SE = standard error calculated in GenAlEx.

[c] Bayesian analyses were run for each field location separately. Admixture proportion (%) = (Number of weakly assigned isolates ($0.5 < q < 0.9$) to NA1 and NA2 + number of isolates with a trichothecene genotype not predictive of subpopulation ($q \geq 0.9$)) / total number of isolates in each trial site * 100.

Table 3-5 Summary of admixture *Fusarium graminearum* isolates from the Brunkild and St. Jean Baptiste AFLP dataset as determined from STRUCTURE analysis with *K*=2.The *q* values for NA1 and NA2 subpopulations are the mean of 10 replicate runs.

Site	Isolate[a]	Trichothecene genotype	Subpopulation assignment	NA1 mean *q* value	NA2 mean *q* value
Brunkild	BR1LS10	15ADON	NA1	0.895	0.105
	BR4LS13	15ADON	NA1	0.893	0.107
	BR4LS4	15ADON	NA1	0.888	0.112
	BR4RS4	15ADON	NA1	0.725	0.275
	BR3RS7	15ADON	NA1	0.503	0.497
	BR4RS11	15ADON	NA2	0.004	0.996
	BR4RS7	15ADON	NA2	0.006	0.994
	BR3US10	15ADON	NA2	0.008	0.992
	BR1LS16	15ADON	NA2	0.009	0.991
	BR3US14	15ADON	NA2	0.010	0.991
	BR3RS2	15ADON	NA2	0.011	0.989
	BR3US13	15ADON	NA2	0.015	0.985
	BR2US5	15ADON	NA2	0.024	0.976
	BR4US15	15ADON	NA2	0.039	0.961
	BR3LS9	15ADON	NA2	0.070	0.930
	BR4RS9	15ADON	NA2	0.37	0.63
	BR3LS1	3ADON	NA2	0.108	0.893
	BR1RS6.1	3ADON	NA2	0.167	0.833
	BR2LS6	3ADON	NA2	0.344	0.656

	BR2RS11.1	3ADON	NA2	0.409	0.591
	BR2US9.1	3ADON	NA2	0.416	0.584
	BR3US16	3ADON	NA1	0.957	0.043
St. Jean Baptiste	SJ1RS3	15ADON	NA1	0.881	0.119
	SJ4US9	15ADON	NA1	0.838	0.162
	SJ1LS14	15ADON	NA1	0.838	0.162
	SJ1RS8	15ADON	NA1	0.662	0.338
	SJ2LS18	15ADON	NA1	0.556	0.444
	SJ2US5	15ADON	NA2	0.007	0.993
	SJ1LS18	15ADON	NA2	0.007	0.993
	SJ4US15	15ADON	NA2	0.008	0.992
	SJ4US5	15ADON	NA2	0.008	0.992
	SJ2US7.1	15ADON	NA2	0.010	0.990
	SJ4LS11	15ADON	NA2	0.012	0.988
	SJ1RS5	15ADON	NA2	0.012	0.988
	SJ1US15	15ADON	NA2	0.012	0.988
	SJ4LS19	15ADON	NA2	0.013	0.987
	SJ3US19	15ADON	NA2	0.019	0.981
	SJ3LS14	15ADON	NA2	0.031	0.969
	SJ2RS10	15ADON	NA2	0.139	0.861
	SJ2US20.1	15ADON	NA2	0.165	0.835
	SJ4US12	15ADON	NA2	0.27	0.73
	SJ3US2	15ADON	NA2	0.339	0.661

SJ1US12	3ADON	NA2	0.13	0.87
SJ2LS17	3ADON	NA2	0.14	0.86
SJ1LS15	3ADON	NA2	0.146	0.854
SJ2US12	3ADON	NA2	0.151	0.849
SJ3RS9	3ADON	NA2	0.171	0.829
SJ2US6	3ADON	NA2	0.187	0.813
SJ1LS3	3ADON	NA2	0.212	0.788
SJ3RS7	3ADON	NA2	0.293	0.707
SJ3RS8	3ADON	NA2	0.356	0.644
SJ2LS2	3ADON	NA2	0.361	0.639
SJ1RS19	3ADON	NA2	0.381	0.619
SJ3RS14.1	3ADON	NA2	0.399	0.601
SJ1LS17	3ADON	NA2	0.473	0.527
SJ2LS15	3ADON	NA2	0.48	0.52
SJ3LS10	3ADON	NA1/NA2	0.499	0.502
SJ2RS18	3ADON	NA1	0.84	0.16
SJ2RS19	3ADON	NA1	0.759	0.241
SJ1US17	3ADON	NA1	0.7	0.3
SJ2RS14	3ADON	NA1	0.553	0.447
SJ2US14	3ADON	NA1	0.523	0.477

[a] Isolates in italics denote isolates with a trichothecene genotype not predictive of subpopulation ($q > 0.5$). All others are 15ADON and 3ADON isolates with a low probability of assignment to the NA1 subpopulation or NA2 subpopulation, respectively ($0.5 < q < 0.9$).

Unbiased gene diversity (*H*) estimates for the NA1 and NA2 subpopulations from the full Brunkild dataset were similar at 0.169 and 0.164, respectively (

Table 3-4). Exclusion of admixtures (isolates with a low probability of assignment to subpopulation ((0.5 < q < 0.9)) from the Brunkild dataset slightly reduced H for both subpopulations to 0.161 (NA1) and 0.159 (NA2). H for the full St. Jean Baptiste dataset was similar to Brunkild for the NA2 subpopulation (0.165); however H for the NA1 subpopulation was higher at 0.197. As would be expected with the larger percentage of admixture isolates in the St. Jean Baptiste dataset, in comparison to the Brunkild dataset, H was reduced substantially for both subpopulations when admixtures were removed from the analysis of the St. Jean Baptiste dataset (0.178 NA1 and 0.150 NA2). H for the St. Jean Baptiste dataset excluding admixtures still remained higher than the Brunkild NA1 subpopulation when admixtures were included (0.178 *vs.* 0.169).

3.4.5 Genetic variation between assigned subpopulations and field fungicide treatments.

AMOVA was performed to investigate how much of the genetic variation could be explained by the subpopulation as assigned by the STRUCTURE analysis (NA1/NA2) and the fungicide field treatment (Table 3-6 and Table 3-7). To investigate the presence of admixtures on the partitioning of genetic variation by subpopulation, two separate analyses were run for each of the Brunkild and St. Jean Baptiste datasets. The first dataset included all isolates and the second dataset excluded admixtures that had a low probability of assignment to either subpopulation, but included recombinants (isolates assigned to subpopulation not predictive of trichothecene genotype) with $q \geq 0.9$. The one isolate (SJ3LS10) from the St. Jean Baptiste dataset that could not be definitively assigned to either subpopulation was omitted from all analyses.

Table 3-6 Analysis of molecular variance (AMOVA) for AFLP data of *F. graminearum* isolates from the Brunkild trial. Isolates were assigned to two subpopulations (NA1/NA2) and collected from three field fungicide treatments (untreated, triazole fungicide applied at recommended timing and triazole fungicide applied at late timing).

Dataset[a]	**Source**	**d.f**[b]	**Sums of Squares**	**Mean Square**	**Variance**	**% of total variance**	**Φ**[c]	***P***[d]
Including Admixtures	Between subpopulations	1	252.7	252.7	3.114	33.808	Φct = 0.337	0.087
	Between treatments within subpopulations	4	23.0	5.7	-0.009	-0.102	Φsc = -0.002	0.681
	Within treatments	223	1361.7	6.1	6.106	66.294	Φst = 0.338	<0.001
	Total	228	1637.4		9.211	100.000		
Excluding Admixtures	Between subpopulations	1	255.1	255.1	3.428	36.427	Φct = 0.365	<0.001
	Between treatments within subpopulations	4	25.3	6.3	0.010	0.101	Φsc = 0.002	0.329
	Within treatments	212	1266.4	6.0	5.974	63.472	Φst = 0.364	<0.001
	Total	217	1546.8		9.412	100.000		

[a] Isolates with a low probability of assignment to subpopulation ($0.5 < q < 0.9$) were removed prior to analysis for datasets excluding admixtures.
[b] Degrees of freedom
[c] Φct is a measure of the extent of genetic differentiation between subpopulations, Φsc is a measure of the extent of genetic differentiation between fungicide treatments within subpopulations, Φst is a measure of the extent of genetic differentiation among isolates within treatments within subpopulations
[d] *P* is probability of obtaining an equal or lower variance component and Φ-statistic determined by on 1000 random permutations of the data sets.

Table 3-7 Analysis of molecular variance (AMOVA) for AFLP data of *F. graminearum* isolates from the St. Jean Baptiste trial. Isolates were assigned to two subpopulations (NA1/NA2) and collected from three field fungicide treatments (untreated, triazole fungicide applied at recommended timing and triazole fungicide applied at late timing).

Dataset[a]	**Source**	**d.f**[b]	**Sums of Squares**	**Mean Square**	**Variance**	**% of total variance**	**Φ**[c]	***P***[d]
Including Admixtures	Between subpopulations	1	239.4	239.4	2.686	29.226	Φct = 0.292	0.099
	Between treatments within subpopulations	4	25.4	6.4	-0.005	-0.050	Φsc = -0.001	0.68
	Within treatments	217	1412.3	6.5	6.508	70.824	Φst = 0.292	<0.001
	Total	222	1677.1		9.190	100.000		
Excluding Admixtures	Between subpopulations	1	252.4	252.4	3.361	36.135	Φct = 0.360	<0.001
	Between treatments within subpopulations	4	22.7	5.7	-0.009	-0.093	Φsc = -0.001	0.738
	Within treatments	189	1124.4	5.9	5.949	63.958	Φst = 0.361	<0.001
	Total	194	1399.4		9.301	100.000		

[a] Isolates with a low probability of assignment to subpopulation ($0.5 < q < 0.9$) were removed prior to analysis for datasets excluding admixtures.
[b] Degrees of freedom
[c] Φct is a measure of the extent of genetic differentiation between subpopulations, Φsc is a measure of the extent of genetic differentiation between fungicide treatments within subpopulations, Φst is a measure of the extent of genetic differentiation among isolates within treatments within subpopulations
[d] *P* is probability of obtaining an equal or lower variance component and Φ-statistic determined by on 1000 random permutations of the data sets.

For the Brunkild dataset including admixtures, the subpopulation accounted for 33.808% of the genetic variation and the level of genetic differentiation (Φct) between NA1 and NA2 was 0.337. However, these values were not significant at the 0.05% significance level based on 1000 permutations (P = 0.087) (Table 3-6). When admixtures were excluded from the analysis, the subpopulation accounted for 36.427% of the genetic variation and the level of genetic differentiation between NA1 and NA2 was Φct = 0.365 which were significant at the 5% significance level. Fungicide treatment was not a significant source of genetic variation whether admixtures were included (-0.102, P = 0.681) or excluded from the analysis (-0.002, P = 0.329).

For the St. Jean Baptiste dataset including admixtures, the subpopulation accounted for 29.226% of the genetic variation and the level of genetic differentiation between NA1 and NA2 was Φct = 0.292. However, this was not significant at the 0.05% significance level based on 1000 permutations (P = 0.099) (Table 3-7). When admixtures were excluded from the analysis, the subpopulation accounted for 36.135% of the genetic variation and the level of genetic differentiation between NA1 and NA2 was Φct = 0.360 which were significant at the 5% significance level. Fungicide treatment was not a significant source of genetic variation whether admixtures were included (-0.001, P = 0.68) or excluded from the analysis (-0.001, P = 0.738).

3.4.6 Discriminant analysis of Principle Components

Discriminant analysis of Principle Components (DAPC) maximises the between group variation, whilst minimising the within group variation. This method may indicate small differences in the genetic variation between treatments that cannot be observed with alternative multivariate data

analyses methods such as AMOVA and principle component analysis. Due to the presence of two distinct genetic clusters/populations (NA1 and NA2), DAPC analysis was performed utilising six *a priori* groups based on the assigned population from STRUCTURE analysis (*i.e.* NA1/NA2 including admixtures) and the field fungicide treatment.

DAPC analysis for isolates from both the St. Jean Baptiste and Brunkild sites clearly demonstrated that the majority of the between group variation occurs between the assigned STRUCTURE populations, as shown on the horizontal axis (Figure 3-10 and Figure 3-11). The second axis represents a small amount of the between group variation compared with the first axis. Within the NA2 population, treatment groups remained clustered together whereas in the NA1 population, the groupings based on field fungicide treatment were more spaced out in both the Brunkild and St. Jean Baptiste datasets.

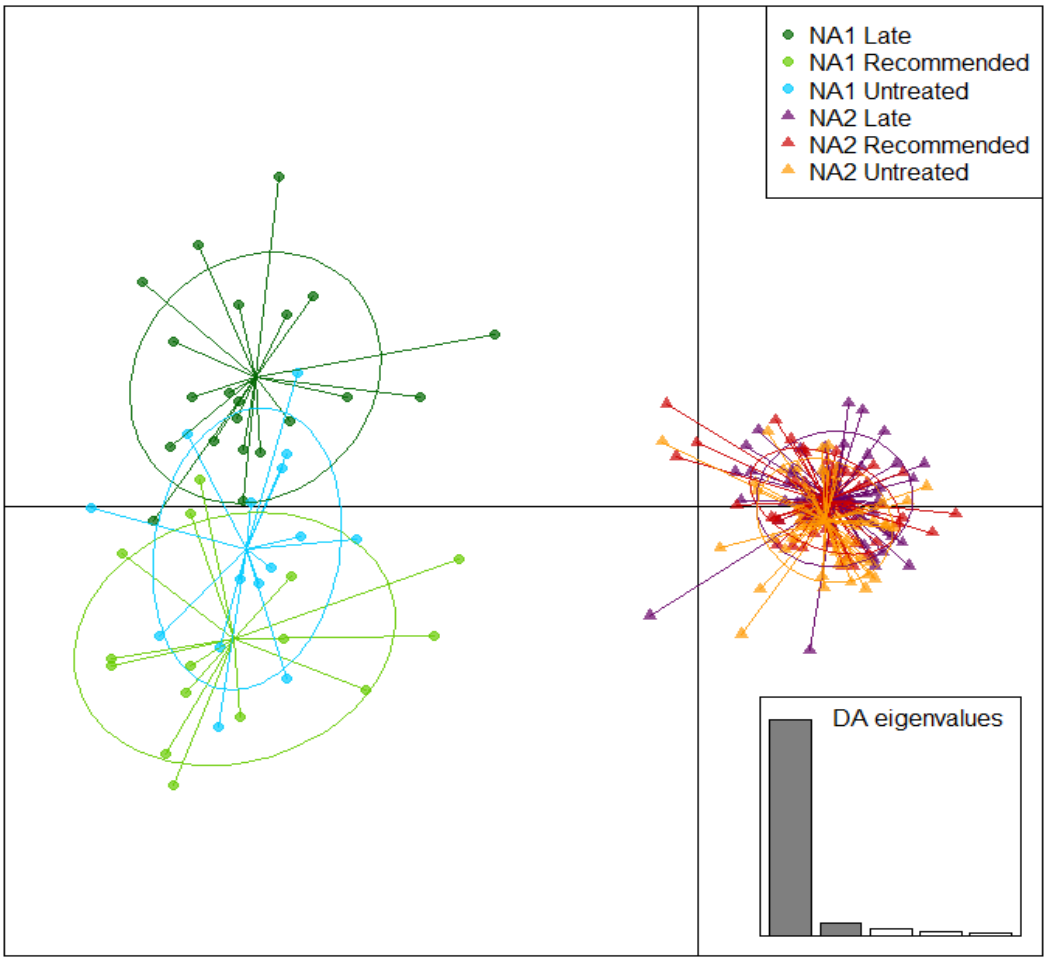

Figure 3-10 Discriminant analysis of principal components (DAPC) for the Brunkild AFLP dataset. The number of axes retained for the principal component analysis was 60 and 2 for the discriminant analysis. Six *a priori* groups were set based on the assigned subpopulation and field fungicide treatment (NA1 and NA2 untreated, NA1 and NA2 triazole fungicide at recommended timing, NA1 and NA2 triazole fungicide at late timing). Points represent individual observations. Lines and shapes and colours represent group membership (subpopulation and fungicide treatment within each subpopulation) as detailed in the legend, (top right). Ellipses represent an analogue of a 67% confidence interval. The insert graph displays the discriminant analysis eigenvalues in relative magnitude with the largest two values in dark grey (bottom right). The first eigenvalue was 737.6; the second eigenvalue was 43.06.

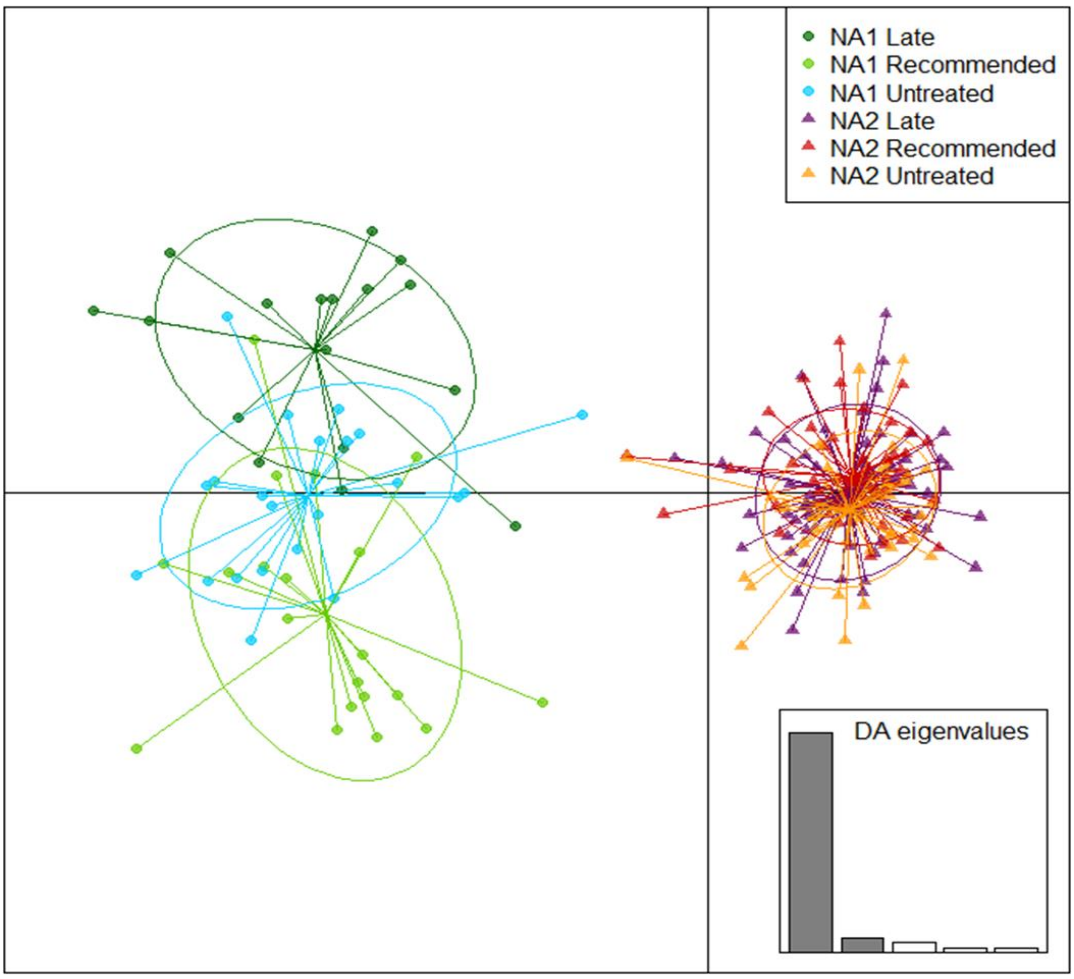

Figure 3-11 Discriminant analysis of principal components (DAPC) for the St. Jean Baptiste AFLP dataset. The number of axes retained for the principal component analysis was 60 and 2 for the discriminant analysis. Six *a priori* groups were set based on the assigned subpopulation and field fungicide treatment (NA1 and NA2 untreated, NA1 and NA2 triazole fungicide at recommended timing, NA1 and NA2 triazole fungicide at late timing). Points represent individual observations. Lines and shapes and colours represent group membership (subpopulation and fungicide treatment within each subpopulation) as detailed in the legend, (top right). Ellipses represent an analogue of a 67% confidence interval. The insert graph displays the discriminant analysis eigenvalues in relative magnitude with the largest two values in dark grey (bottom right). The first eigenvalue was 463.5; the second eigenvalue was 30.63.

3.5 Discussion

The overall aim of this study was to evaluate the response of field populations of *F. graminearum* to fungicide application by using AFLP markers to assess for differences in the genetic variation in isolates sampled from fields with fungicide treated and untreated areas. This study provided sufficient data to successfully address that objective, while also providing a more general field population level understanding of *F. graminearum* in the Red River Valley region of Manitoba, building on the current understanding of population dynamics in this area. The discussion of the results will be presented in three sections. First, the field trial data will be addressed, followed by the results on the population structure inferred from the AFLP marker data from the sampled isolates, and then concluding with the effect of fungicide use on the genetic variation observed from the sampled isolates.

3.5.1 Field study FHB levels

The two sample sites were in close proximity geographically and as such, the climate data for both sites was broadly similar. Soybean was the previous crop for both sites, indicating that infected residue levels were low (Dill-Macky and Jones, 2000). A deeper history of preceding crops and tillage practices at each site is unknown and could affect inoculum levels on a field-by-field basis. The only known difference between sites was the variety used, with a more susceptible variety in St. Jean Baptiste (AAC Elie, intermediate level resistance) than Brunkild (AAC Brandon, moderate resistance). The relatively dry summer in 2017 meant that FHB levels were low compared to previous years; epidemic levels of FHB were recorded across Manitoba in 2008-2010 and 2016 (Canadian Grain Commission, 2017a; Tittlemier *et al.*, 2019). This is reflected in the field FHB index data and the analysis of FUS DMG and DON in the harvested

grain samples. Brunkild had a higher FHB index than St. Jean Baptiste; however, this only represents a small difference in the context of a low disease year. The harvested samples corroborated the overall low disease levels observed in the field; however, the data contradicted the field observation of higher disease levels at the Brunkild site as compared to the St. Jean Baptiste site. This could be due to loss of FDKs in the field at harvest as lighter FDKs can be blown out of the combine depending on the settings, thus reducing the percentage of diseased material in harvested samples (McMullen *et al.*, 2012).

While 2017 was a low disease year, there was evidence from both trial sites that the application of fungicide reduced FHB levels in comparison to the untreated plots. This was evident from the FHB disease levels in the field for both sites (Figure 3-1). The presence of mouldy kernels in the St. Jean Baptiste harvested grain sample from the untreated plots reduced the grain grade to feed level (Table 3-3). The high DON content in this sample suggests the mouldy kernels were in part due to *Fusarium* infection. DON was not detected in the fungicide treated plots and their grain grades were not affected by the presence of FDKs. This supports the economic value in applying a fungicide to control FHB. However, as mentioned above, this effect was not observed in the Brunkild harvest samples with 0% FUS DMG, even in the untreated plots. The timing of fungicide application did not impact the overall efficacy, suggesting that growers may have more flexibility in when to apply a fungicide for FHB control. However, this was not a main objective of this study and data from more locations and years would be required to fully support this precursory assessment.

3.5.2 Population dynamics of *F. graminearum* in sampled fields

The field study sites were purposefully kept as close to natural agronomic environment as possible. Trials were located in non-research fields, whereby only natural inoculum sources had previously occurred (*i.e.* no previous artificial inoculation) and only natural infection was used in the year of study. Cropping practices such as variety choice, seeding date, and fungicide type were selected by the grower, with only the prerequisite of fungicide treated and untreated areas set by Manitoba Agriculture. While these parameters mean that inferences regarding the *F. graminearum* population in the study area can be made (*i.e.* the Red River Valley of Manitoba), the recovery of isolates, from both fungicide treated and untreated areas, may affect the analyses of these individual field populations.

An AFLP marker approach was applied to isolates recovered from sampled wheat spikes to enable the assessment of genetic diversity (*i.e.* gene and genotype diversity), population structure, and the impact of fungicide treatment on these variables at the field level. High haplotype diversity was confirmed at the field level for both study sites. There was a low level of genetic differentiation between the sites, with most genetic diversity distributed within sites. These population characteristics are in concurrence with previous *F. graminearum* population in North America (Dusabenyagasani *et al.*, 1999; Zeller *et al.*, 2003, 2004; Mishra *et al.*, 2004; Fernando *et al.*, 2006; Gale *et al.*, 2007; Guo *et al.*, 2008). The high genotypic diversity and minor differentiation between field populations observed in the study and others is indicative of a large population size, sexual recombination, frequent gene flow, and thus a high adaptive capacity (Miedaner *et al.*, 2008). Interestingly, more isolates of the same genotype were found across the two sites than within. This may indicate a greater role of wind-dispersed ascospores

produced by homothallic reproduction than asexually produced macroconidia as primary inoculum across the Red River Valley.

Previous population studies have described the presence of two sympatric sub-populations of *F. graminearum* in the parts of North America, including the western Prairie Provinces (Gale *et al.*, 2007; Ward *et al.*, 2008; Kelly *et al.*, 2015; Liang *et al.*, 2015). These are described as NA1 and NA2, with NA1 encompassing an endemic population dominated by 15ADON genotypes and a more recently introduced NA2 population mainly composed of 3ADON genotypes. Previous studies have shown significant differences in phenotypic traits for these populations, including pathogenicity on wheat, *in planta* and *in vitro* trichothecene accumulation, and *in vitro* growth and sporulation (Ward *et al.*, 2008; Puri and Zhong, 2010; Foroud *et al.*, 2012). As such, it was deemed essential for this study to investigate population substructure prior to further analysis of the effect of fungicide application in the field.

The overall proportion of trichothecene genotypes for isolates collected from both fields were similar at 27.3 % 15ADON and 72.7% 3ADON in Brunkild and 30.3% 15ADON and 69.7% 3ADON in St. Jean Baptiste. These proportions agree with the values reported from isolates collected from wheat in Manitoba in 2004/2005 (Guo *et al.*, 2008), between 2005 and 2007 (Kelly *et al.*, 2015), and in harvest surveys by the Canadian Grain Commission from 2011-2017 (Canadian Grain Commission, 2017b). Although this study covers a small geographical area, when considered in combination with the reported values it suggests the rapid increase in 3ADON frequency, as was observed in Manitoba from 1998 to 2004 by Ward *et al.*, (2008), may be stabilising around current levels. The reasons for the increase in 3ADON frequency still

remains unclear, however, such stabilization indicates the 15ADON chemotype population has direct or indirect fitness traits that enable its persistence in the population. In contrast with Western Canada, the 15ADON chemotype remains prevalent in Ontario, with observed frequencies ranging from 93% to 100% (Tamburic-Ilincic *et al.*, 2006, 2015; Amarasinghe *et al.*, 2009). There is speculation that the high corn production in Ontario compared with the Western provinces may be a contributing factor to these differences in chemotype frequencies (Tamburic-Ilincic *et al.*, 2015). The acreage of corn has been increasing in Manitoba, and therefore may be supporting the persistence of the 15ADON chemotype. No isolates with the NIV or recently discovered NX-2 genotype were recovered. This is not surprising given the low frequency reported in previous studies in the Midwestern US and Western Canada (Liang *et al.*, 2014; Kelly *et al.*, 2015).

STRUCTURE analysis of the AFLP data from this study supported the presence of two subpopulations at each site that corresponded to the NA1 and NA2 populations that strongly associate with the 15ADON and 3ADON trichothecene genotype respectively (Liang *et al.*, 2014; Kelly *et al.*, 2015). However, the presence of admixtures provides evidence of recombination between these two subpopulations and a breakdown of the chemotype / subpopulation association in the area studied. In this study the percentage of admixtures was almost twice as high in the St. Jean Baptiste site than Brunkild. This could indicate greater levels of recombination between NA1 and NA2 in the St. Jean Baptiste site. Alternatively, admixtures may be at the same frequency in primary inoculum at both sites, but fitness traits in these isolates may be better suited to the St. Jean Baptiste trial resulting in a greater number being recovered from infected spikes. The primary difference between sites was the variety grown, with a variety

with an intermediate FHB resistance level in St. Jean Baptiste and a variety with a moderate resistance level in Brunkild. Admixtures may have incurred a fitness penalty due to the more tolerant variety grown at Brunkild, resulting in a lower frequency of recovered admixtures.

The higher admixture percentage in the isolates from St. Jean Baptiste reduced genetic differentiation (Φct) between NA1 and NA2 compared to Brunkild, however, Φct remained high indicating these subpopulations are still highly differentiated (Table 3-6 and Table 3-7). Φct between NA1 and NA2 was only statistically significant when admixtures were excluded from the analysis for both sites. The insignificant Φct values indicate a reduced level of genetic differentiation between the NA1 and NA2 subpopulations compared to a previous study using isolates recovered between 2005 and 2007 in Manitoba (Kelly *et al.*, 2015). Furthermore, the percentage of admixture isolates with weak assignment ($q < 0.9$) was lower in those isolates at 2.5%, compared to 4.8% (Brunkild) and 12.9% (St Jean Baptiste) in this study. While these observations indicate increased recombination between these subpopulations overtime, differences in the spatial sampling scale between these studies means further studies are required to confirm this.

Analysis of the number of isolates with trichothecene genotype not predicted by population identity (recombinants) demonstrated a directional bias in selection for the NA2 genetic background, with a larger proportion of 15ADON isolates with a NA2 membership, but rarely 3ADON with NA1 membership in both sites. The directional bias is in agreement with surveys from Western Canada and the Upper Midwestern US and suggests a fitness advantage of NA2 over NA1 in this geographical area (Liang *et al.*, 2014, 2015; Kelly *et al.*, 2015). In the study by

Kelly *et al.*,(2015), 33% of 15ADON trichothecene genotypes were assigned to NA2 while only 1% of 3ADON trichothecene genotypes were assigned to NA1, demonstrating a higher level of such recombinants compared to this study. However, it should be noted that the isolates from the former study cover a wider geographical range, and therefore the regional differences in the percentages of recombinants could be a factor in these discrepancies. In combination with the observed plateau in the frequency of 3ADON trichothecene genotypes in the area, the directional bias in the recombinants (15ADON with NA2 background) suggests that chemotype alone has not provided the selective advantage that has resulted in the predominance of the 3ADON trichothecene genotypes in Manitoba.

The population dynamics of *F. graminearum* may be affected by the host prevalence in a region. Higher levels of genetic differentiation between NA1 and NA2 subpopulations in combination with lower frequencies of admixtures were observed in isolates recovered from barley compared to isolates recovered from wheat in the Upper Midwestern U.S, indicating different rates of recombination between these subpopulations based on host (Liang *et al.*, 2015). As previously discussed, the increase in corn acreage in Manitoba could be a contributing factor in the apparent stabilising of trichothecene genotypes in the province. An analysis of isolates from Ontario, where 15ADON is the most prevalent genotype and corn is widely grown, admixtures demonstrated an opposite bias of genetic background and trichothecene genotype with 25% 3ADON isolates assigned to NA1 and only 5% 15ADON isolates assigned to NA2 ((Kelly *et al.*, 2015). While these isolates were recovered from wheat, the authors speculated that corn production may be affecting the population dynamics (*i.e.* selecting for NA1 background) (Kelly *et al.*, 2015). A study by Kuhnem *et al.*, (2015) demonstrated that trichothecene genotype

frequencies did not differ between isolates recovered from corn ears, corn residues, wheat spikes, and the atmosphere in New York State, indicating that the host is not affecting the population structure. However, this study did not assess the genetic populations of these sampled isolates, so selection for a genetic background by host is unknown. Further population genetic studies across a range of crops is required to better understand the influence of host prevalence on the population dynamics of *F. graminearum* in Manitoba.

Gene diversity was also measured in the two subpopulations. The study by Kelly *et al* (2015) indicated the NA1 population has higher gene diversity than the NA2 population in Manitoba. Evidence supports the theory that the NA2 subpopulation was recently introduced to North America, thus resulting in a lower genetic diversity due to the founder effect (Gale *et al.*, 2007). The gene diversity of the NA1 subpopulation in St. Jean Baptiste was higher than the NA2 subpopulation (

Table 3-4). However, in the Brunkild isolates, the gene diversity of the NA1 subpopulation was similar to the NA2 subpopulation, which in turn, was similar to the St Jean Baptiste NA2 subpopulation. The comparable gene diversity of NA1 and NA2 in Brunkild may indicate that NA2 diversity is increasing, which would not be unexpected over the time since it was introduced to North America. However, the lack of consensus observed in the same time period between the geographically close sites of this study is unexpected. Reasons for these differences could include an impact of local agronomic factors on the diversity of the primary inoculum for each site, or the effect of the wheat variety on the diversity of recovered isolates. If the latter, it would suggest the moderately resistant variety is reducing gene diversity of the NA1

subpopulation. Interestingly, all isolates of the same genotype had NA2 background, pointing to lower levels of genotypic diversity in the overall NA2 subpopulation.

In summary, the AFLP data provided a detailed understanding of the population dynamics in the two study sites. Many characteristics are in accordance with the existing body of literature covering Western Canada. This indicates that the sampling strategy and marker type used provided a good level of resolution to understand the population dynamics in the study sites. The discrepancies between this study and other studies for some of the population characteristics have raised questions around the impact of the agro-environment and temporal changes in *F. graminearum* populations. Addressing some of these inconsistencies with larger scale studies across multiple host crops would improve the knowledge of *F. graminearum* population dynamics in Manitoba and the impact of current management strategies.

3.5.3 Effect of fungicide treatments on the genetic variation of *F. graminearum* field populations

The observation that no isolates of the same genotype were found within field treatments suggests fungicide treatment did not select for a specific genotype. However, given the high genotypic diversity within study sites, this is not an unexpected outcome. The trichothecene genotype ratios were also not affected by field treatment and were close to expected 75% 3ADON to 25% 15ADON frequencies based on recent surveys in Manitoba (Canadian Grain Commission, 2017b). To the best of the author's knowledge, there are no previous studies that fully investigate the effect of fungicide application on the recovery of *F. graminearum* chemotype ratios from spring wheat. As such, no direct comparison to the existing literature can

be made. A study by Amarasinghe *et al.* (2013) showed that in artificially inoculated spring wheat field trials, all six isolates of either 15ADON or 3ADON genotypes responded to fungicide use and there was no fungicide by isolate interaction. While a small sample number of isolates were used, the study indicated that these trichothecene genotypes responded equally to fungicides. However, the study used a solo inoculum of each isolate, so competition and recovery of isolates was not investigated. In the current study, natural inoculum was used which presents study of a system closer to the real-world environment, although with multiple known and unknown variables which can reduce the power to detect such an effect. The use of artificial inoculum in the field could provide clearer evidence of the impact of fungicide on the recovery of specific genotypes; however, the role of naturally occurring ascospores as primary inoculum has made inferences from such field studies difficult (Gilbert *et al.*, 2014).

The AMOVA takes into account all of the genetic variation among and between groups (*i.e.* the NA1/NA2 subpopulation and fungicide field treatment). This showed that there was no significant genetic differentiation between different field fungicide treatments (Table 3-6 and Table 3-7). This analysis was performed both in the presence of and absence of admixtures: the results were similar for both analyses. Accordingly, the results of this study provide no evidence that fungicide treatment has a significant effect on admixtures. However, the low numbers of admixtures due to sampling meant insufficient power to detect any such effects.

The DAPC analysis was used as an approach to visualise the data whereby any between group differences in genetic variation were maximised and within group variation minimized. In independent analyses of both sites there was a small amount of genetic differentiation between

fungicide treatments in the NA1 subpopulation, whereas the NA2 fungicide treatment groups clustered close together (Figure 3-10 and 11). These analyses should be considered with some caution as they could be prone to the low sample size of the NA1 subpopulation. Further studies are required to better understand this pattern, using a larger sample size and over a range of locations and years. If this result proves repeatable, it could indicate that the NA1 subpopulation is under greater selection pressure from the fungicide application than the NA2 subpopulation. This may also provide a partial explanation of why the 3ADON genotype has proliferated in western provinces. There is evidence that the NA2 subpopulation originated from Europe, where the population will have experienced different agro-environments, including possibly greater fungicide exposure, thus affecting its fitness in the new environment (Ward *et al.*, 2008). This is supported by genomic analysis of North American NA1 and NA2 isolates that has identified regions of the genome with signatures of selection, including fungicide tolerance (Kelly and Ward, 2018).

3.6 Conclusion

The aim of this study was to understand the effect of triazole fungicide use on the population structure of *F. graminearum*. Molecular AFLP analysis found similar population characteristics to previous analyses in Western Canada, including in Manitoba. Inconsistencies between studies in respect to admixture frequencies and gene diversity in NA1 and NA2 may be attributable to the difference of sampling scale between this field level study and province level studies. Taking the field level population structure into account, there was no clear evidence that fungicide treatment affected *F. graminearum* population structure, although the DAPC analysis may indicate some minor differentiation among fungicide treatments in the NA1 subpopulation.

However, due to the low frequency of NA1 isolates in the field, the strength of this analysis is weak. The NA2 subpopulation did not show any population differentiation based on fungicide treatment. Given the high frequency of the NA2 subpopulation, the results of this study indicates that fungicide application is not significantly impacting the population structure of the majority of the *F. graminearum* population in Manitoba.

This study was limited to a specific geographical area in a single growing season, and therefore cannot provide an understanding of longer term effects of integrated pest management strategies on the *F. graminearum* populations. Further studies on a larger geographical scale with more isolates to increase the NA1 sample numbers and over several growing seasons would be needed to further investigate the impact of fungicide application on *F. graminearum* populations in Manitoba.

4 INVESTIGATING THE PHENOTYPIC VARIATION IN FUNGICIDE SENSITIVITY OF *FUSARIUM GRAMINEARUM* FIELD ISOLATES

4.1 Abstract

Triazole fungicides are a key component of an Integrated Pest Management (IPM) strategy for the control of FHB. However, variation in the efficacy of fungicides in the field has been observed and is a key concern for growers. The large genetic variation present in *Fusarium graminearum* field populations may be a contributing factor to the variable response to fungicides. In this study, the phenotypic variation in the sensitivity of isolates, sampled from natural field populations with fungicide treated and untreated areas, to a 1:1 mixture product of prothioconazole and tebuconazole was assessed through the use of a high throughput microtiter-plate based *in vitro* assay. Sensitivity of isolates, measured as the effective concentration to cause 50% growth inhibition (EC_{50}), ranged from EC_{50} 0.11 to 0.46 mg L^{-1}. The mean EC_{50} of isolates recovered from areas with fungicide application at two timings and no-fungicide control did not differ significantly. No link between the genetic data generated by AFLP markers (presented in Chapter 3) and *in vitro* fungicide sensitivity was identified, demonstrating the absence of distinct genetic groups with a unique fungicide sensitivity level in the populations sampled. This result indicates that fungicide application is not acting as a significant selective force on *F. graminearum* populations in a single growing season. A significant field study site by NA1/NA2 subpopulation interaction was observed, with a higher mean EC_{50} in the NA2 subpopulation compared to the NA1 subpopulation from isolates recovered from one of the two trial sites. These data indicate other agronomic factors may be contributing to the selection of isolates within the NA2 subpopulation with a lower sensitivity to triazole fungicides.

4.2 Introduction

An IPM approach is required for adequate control of FHB, with an emphasis on the use of resistant varieties and the application of DeMethylation Inhibitor (DMI) (predominantly the triazole class) foliar fungicides (McMullen *et al.*, 2008; Gilbert and Haber, 2013). However, the efficacy of the triazole fungicides can be highly variable and therefore unpredictable in terms of return on investment for the grower (Paul *et al.*, 2008; Wegulo *et al.*, 2013). The variability of fungicide efficacy on visual disease symptoms and DON may be attributed a number of factors, including poor fungicide coverage, fungicide application timing and weather conditions during grain filling following fungicide application (Paul *et al.*, 2008). It is hypothesised that high genotypic diversity and phenotypic variation in fungicide sensitivity could lead to local adaption to fungicide use in field populations which may also be a contributing factor to the variability in triazole fungicide efficacy (Miedaner *et al.*, 2008; Talas and Mcdonald, 2015).

Since the introduction of DMIs in the 1970's, shifts in the sensitivity of many phytopathogenic fungi to these molecules have been observed (Ma and Michailides, 2005). Triazole fungicides have been increasingly used to control FHB in North America since the 1990's. This has been largely due to improvements in the intrinsic potency of the chemistries, but also due to growing disease pressure (Mcmullen *et al.*, 1997; Morton and Staub, 2008; Wegulo *et al.*, 2013). *In vitro* exposure of a *Fusarium graminearum* strain to sub-lethal concentrations of tebuconazole has demonstrated the capacity of the species to adapt to fungicide use (Becher *et al.*, 2010). Sensitivity shifts in *F. graminearum* have been observed in Germany (Klix *et al.*, 2007) and China (Sun *et al.*, 2014). DMI resistant isolates have been recovered in China and the U.S. (Gale *et al.*, 2002; Yin *et al.*, 2009; Spolti *et al.*, 2014). These observed adaptions of *F. graminearum*

populations are a concern for the future sustainability of fungicide use, particularly as no new chemistries marketed for FHB control have been introduced since 2002 (Morton and Staub, 2008).

In Chapter 3, high levels of genetic variation were observed in 453 *F. graminearum* isolates collected from two CWRS fields in Manitoba, which is consistent with previous reports (Gale *et al.*, 2002; Zeller *et al.*, 2004; Liang *et al.*, 2014; Kelly *et al.*, 2015). However, there was no significant evidence to support the effect of triazole fungicide treatment in the field on the observed genetic variation. In order to gain further understanding from the findings in Chapter 3, the objectives of this study were two-fold. First, to assess the level of phenotypic variation in the *in vitro* sensitivity of isolates to the fungicide applied in the field and whether fungicide application affected this variation among isolates. Second, to use the genetic data from Chapter 3 to investigate the presence of distinct genotypes with varying levels of *in vitro* fungicide sensitivity. A high throughput microtiter-plate based assay was developed to enable screening of the large number of isolates for fungicide sensitivity.

4.3 Materials and Methods

4.3.1 Isolates and inoculum preparation.

All 229 isolates recovered from the Brunkild field trial and 224 isolates recovered from the St. Jean Baptiste trial described in Section 3.4.3 were tested for *in vitro* sensitivity to a 1:1 mixture of tebuconazole and prothioconazole (Prosaro® 250 EC, Bayer, Leverkusen, Germany). Conidia from *F. graminearum* isolates were harvested from 10 day old synthetic nutrient media culture

plates grown in the same conditions as Section 3.3.2. Plates were flooded with 5 ml of 10% sterile glycerol solution and the surface was gently rubbed with a sterile metal spatula. The suspension was filtered through four layers of cheesecloth and then assessed with a Neubauer haemocytometer and adjusted to 2.5 x 10^4 conidia / ml. The 1 ml aliquots were stored in a 10% glycerol solution at -145°C until use in experiments. The conidia concentration in aliquots was optimised based on the final conidia concentration selected for the microtiter plate assay (Appendix 7.2), allowing three technical replicates to be set up from a conidia single aliquot.

4.3.2 Microtiter plate assay

A 96-well plate layout was used to enable testing of the large number of isolates. The assay was optimised via iterations of culture media, spore rate and test durations. A full description of the assay development and validation process is presented in Appendix 7.2 for completeness. The finalised method used was as follows:

Commercial Prosaro® 250 EC fungicide was serial diluted (fivefold) in potato dextrose broth (PDB) (Difco Laboratories, MI, US) in order to obtain seven final test concentrations of 0.0077, 0.384, 0.192, 0.96, 4.8, 24 and 120 mg L^{-1} of a 1:1 tebucaonazole and prothioconazole mixture. Each concentration refers to the amount of each active ingredient, *i.e.* a dose rate of 120 mg L^{-1} is 120 mg L^{-1} of prothioconazole plus 120 mg L^{-1} of tebuconazole. A Prosaro® stock concentration of 1212.8 mg L^{-1} was prepared for each experiment and filter sterilised prior to use for the serial dilutions. A volume of 180µl of each fungicide concentration was added to rows A-G of a 96-well flat-bottomed microtiter plate (Cellstar®, Griener Bio-One, Kremsmünster, Austria). 180µl of PDB containing no fungicide was added to row H as a no-fungicide control.

Frozen 1ml aliquots of conidia suspensions were thawed at room temperature. 20 µl of conidia were added to each plate well to achieve a final concentration of 2.5 x 10^3 conidia per ml. Three technical replicates of each isolate and fungicide concentration and no-fungicide control were set up for each experiment. The plates were sealed with a gas-permeable, breathable rayon film (VWR, PA, US) and incubated in darkness at 22˚C for 72 hours. Optical density was then read at 650 nm in a SpectraMax 340 plate reader (Molecular Devices, CA, US). Each isolate was tested twice in a separate experiment starting from a single conidial aliquot stored at -145°C.

4.3.3 Data Analysis

Data were exported, organized and arranged for analysis in Microsoft Excel. The dose-response relationship was assessed for each strain in each experiment using a log-logistic four parameter model provided by the 'drc' package in R and the effective concentration of tebuconazole and prothioconazole corresponding to 50% growth inhibition (EC_{50}) relative to the maximum and minimum growth was estimated (Ritz *et al.*, 2015). Density distribution curves were performed in R using the "ggpubr" package (Kassambara, 2018). Analysis of variance (ANOVA) using type III sums of squares to assess for the effect of field study site, subpopulation (NA1/NA2), field fungicide treatment, and all combinations of interactions of these factors on the variability of log transformed EC_{50} values among isolates was performed using the 'Car' package in R (Weisberg and Fox, 2011). Diagnostic plots were performed in R. Principle coordinates analysis (PCoA) was performed on the AFLP genetic data presented in Chapter 3 in Genalex 6.2 (Peakall and Smouse, 2006). A DAPC analysis was performed to further assess the genetic variation

among groups of isolates based on the fungicide sensitivity levels determined from the *in vitro* in the R package *adegenet* (Jombart *et al.*, 2010).

4.4 Results

4.4.1 *In vitro* sensitivity of *F. graminearum* isolates to prothioconazole and tebuconazole mixture

All 229 isolates from Brunkild and 224 isolates from St. Jean Baptiste described in Section 3.4.3 were screened for sensitivity to the commercial product Prosaro® (a 1:1 mixture product of tebuconazole and prothioconazole) in a microtiter plate based assay. The EC_{50} values ranged from 0.11 to 0.46 mg L^{-1} (~ 4-fold difference). The density distribution of EC_{50} values for isolates split by site, subpopulation inferred by STRUCTURE analysis (Section 3.3.4) and fungicide treatment in the field are presented in Figure 4-1. The density distribution curves demonstrated that the number of isolates sampled provided a good representation of the population sensitivity range as characterised by the smooth lines, and tails at the ends of the curves. The slightly more irregular curves observed with in the NA1 subpopulation are attributable due to the lower sample numbers in each fungicide treatment within NA1 subpopulations across sites (range 15-22 isolates) compared to the sample numbers in each fungicide treatment within the NA2 subpopulations (range 51-62 isolates).

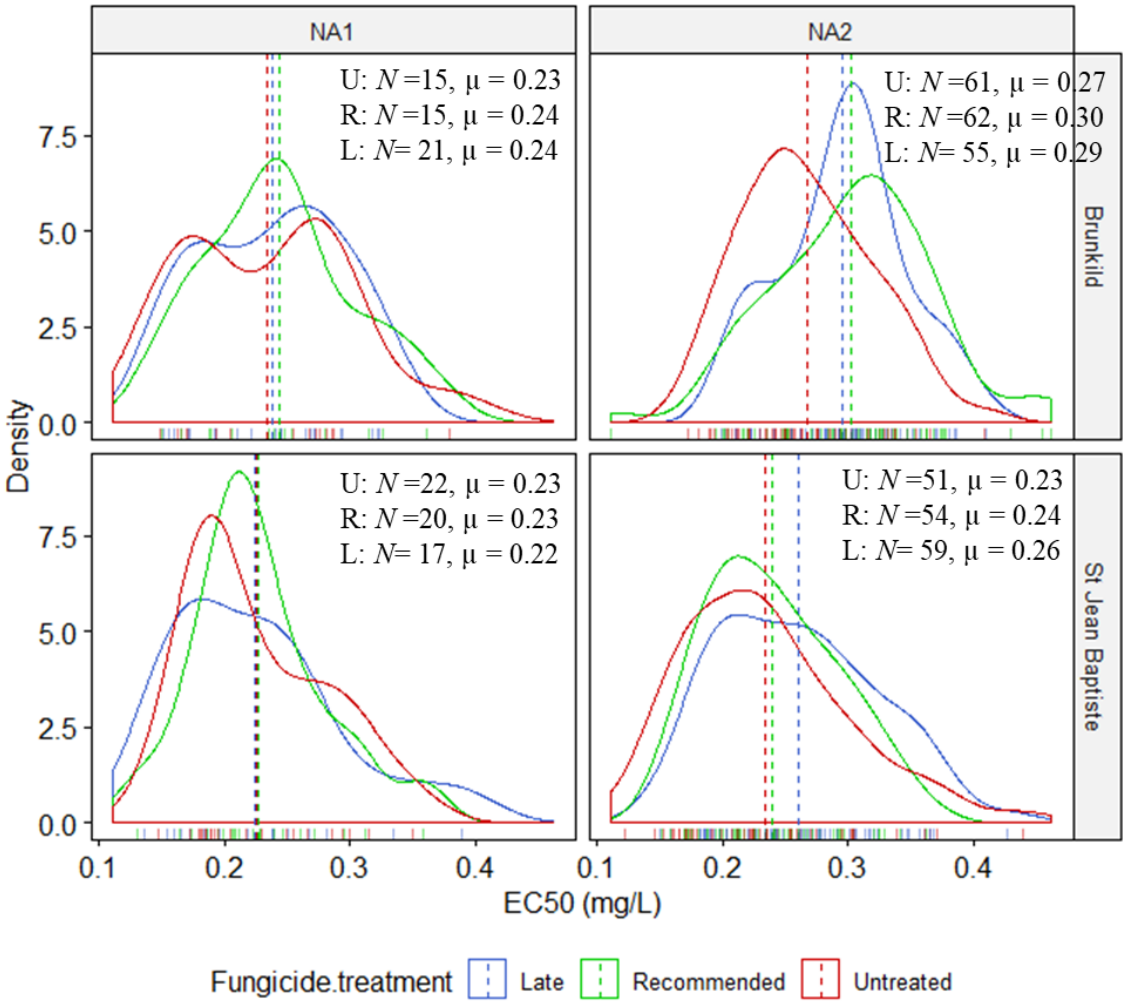

Figure 4-1 Density distribution of EC_{50} (mg L^{-1}) of *F. graminearum* isolates to 1:1 mixture tebuconazole and prothioconazole. Isolates were sampled from one of three field treatments as indicated by line colour: red = no-fungicide control (Untreated), green= fungicide applied at recommended timing (Recommended) and blue = fungicide applied at late timing (Late). Panels are split by the two field study locations (Brunkild and St. Jean Baptiste) and two subpopulations (NA1 and NA2) determined from analysis of AFLP marker data for isolates. Dashed vertical lines represent the mean EC_{50} for each density curve. The lines below each density curve represent individual isolates, colour coded by fungicide treatment. Graph inserts show number of isolate (*N*) used to generate each curve and the mean EC_{50} value (μ) for each curve.

An ANOVA was performed to test the main effects of site, subpopulations and fungicide treatment and the presence of interactions between them. Exploration of the ANOVA performed on log transformed EC_{50} values confirmed normality of the data and homogeneity of variance required to meet the assumptions of the ANOVA (Appendix 7.2: Figure 7-6 and Figure 7-7). ANOVA showed that both site and subpopulation had a significant effect on the mean logEC_{50} and there was a significant interaction between these two factors (Table 4-1). All other factors and interactions were not significant. The interaction plot for the investigation of the significant interaction between site and subpopulation showed that at both sites the NA2 subpopulation had a lower sensitivity (higher mean EC_{50}) compared to the NA1 subpopulation (Figure 4-2). However, the steeper gradient of the line connecting the mean EC_{50} across subpopulations within the Brunkild trial site shows a greater effect of subpopulation on the mean EC_{50} than in the St. Jean Baptiste trial site. The mean EC_{50} values were similar in the NA1 subpopulation in Brunkild (0.24 mg L^{-1}) and both subpopulations in the St. Jean Baptiste study site (0.23 mg L^{-1} and 0.25 mg L^{-1} for NA1 and NA2, respectively). However site the mean EC_{50} for NA2, in the Brunkild study was higher than all of the former at 0.29 mg L^{-1}.

Table 4-1 Analysis of variance (type-III sums of squares) of log EC_{50} to a 1:1 mixture tebuconazole and prothioconazole of *F. graminearum* isolates collected from different fungicide treatments in two field study sites that were assigned to two subpopulations based on AFLP genetic data.

Source	**DF**	**Sum of squares**	**Mean Square**	**F value**	***P*>F**
Site (S)	1	0.2000	0.2000	19.6972	1.15E-05***
Subpopulation (SP)	1	0.3060	0.3060	30.2155	6.56E-08***
Fungicide treatment (F)	2	0.0390	0.0195	1.9459	0.1441
S*SP	1	0.0560	0.0560	5.5540	0.019*
SP*F	2	0.0310	0.0155	1.5174	0.2204
S*F	2	0.0100	0.0050	0.4825	0.6176
S*SP*F	2	0.0050	0.0025	0.2582	0.7725
Residuals	440	4.4610			

* = significant at $P = 0.05$, *** = significant at $P = 0.001$.

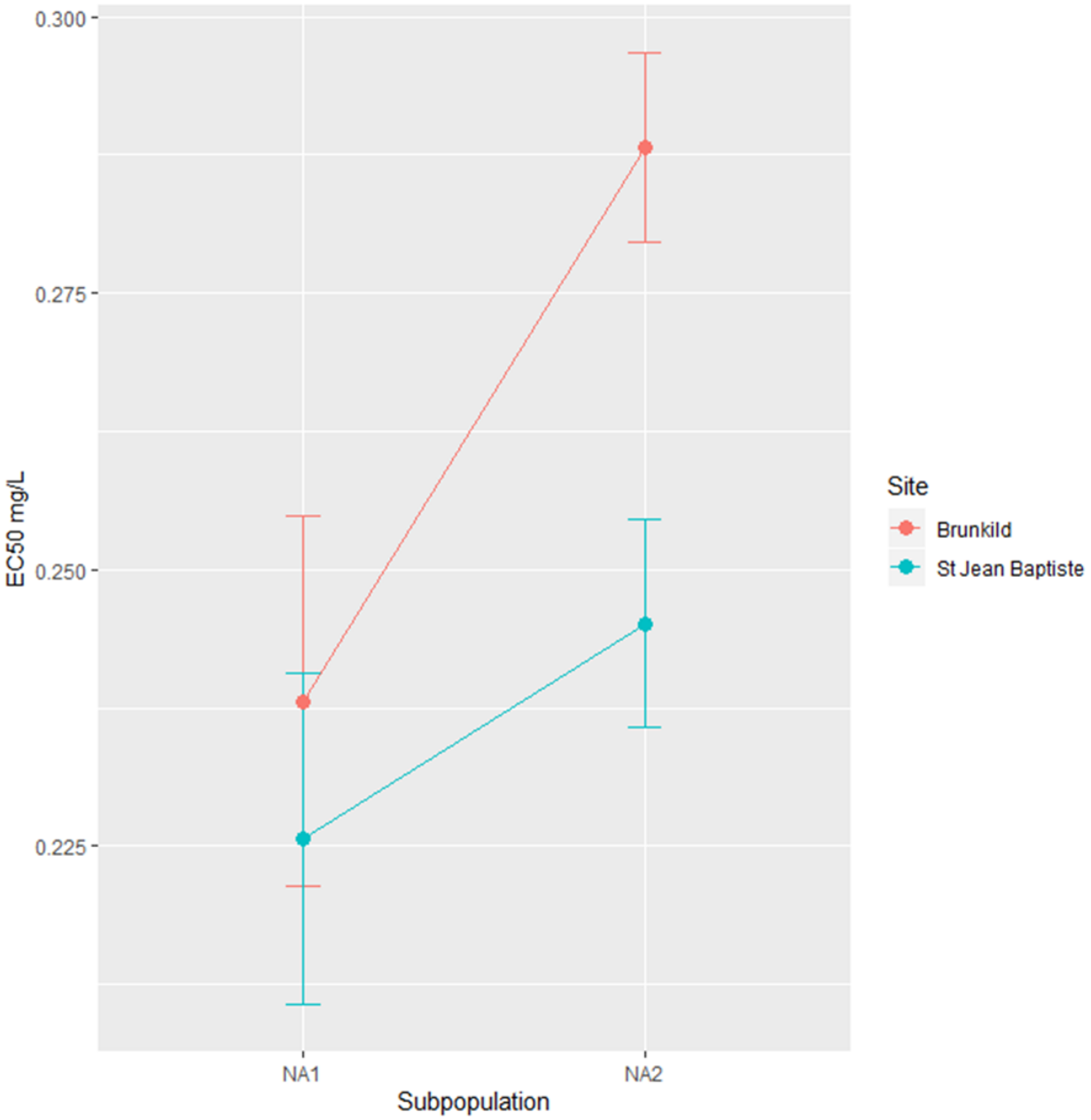

Figure 4-2 Interaction plot of subpopulation (NA1 / NA2) and field trial site (Brunkild / St. Jean Baptiste) on mean EC_{50} values of *F. graminearum* isolates to a 1:1 mixture of tebuconazole and prothioconazole. . Error bars represent 95% confidence intervals.

4.4.2 Exploration of fungicide sensitivity and AFLP data

The *in vitro* sensitivity of isolates to the fungicide mixture and AFLP genetic data was assessed by PCoA. As expected from the analysis of the data in Chapter 3, the first axis clearly separated isolates into two clusters corresponding to the NA1 and NA2 subpopulations (Figure 4-3). Admixtures were included in the analysis which explains some overlap of these clusters. Fungicide sensitivity levels were distributed randomly along the two coordinates. All seven isolates with the lowest sensitivity range (0.4 mg $L^{-1} \leq EC_{50} < 0.5$ mg L^{-1}) were found among NA2 and did not form a distinct group. However, four of these isolates could be split into two pairs that were genetically similar. The first pair was found across the two sites in different fungicide treatments (BR2US9.2 / SJ4LS18.1), while the second pair was found within the same field plot (BR4RS17 / BR4RS11). The DAPC analysis pointed to no major genetic differentiation based on the sensitivity range of isolates, as evident with the large amount of overlap among groups based on the sensitivity ranges within each subpopulation (Figure 4-4).

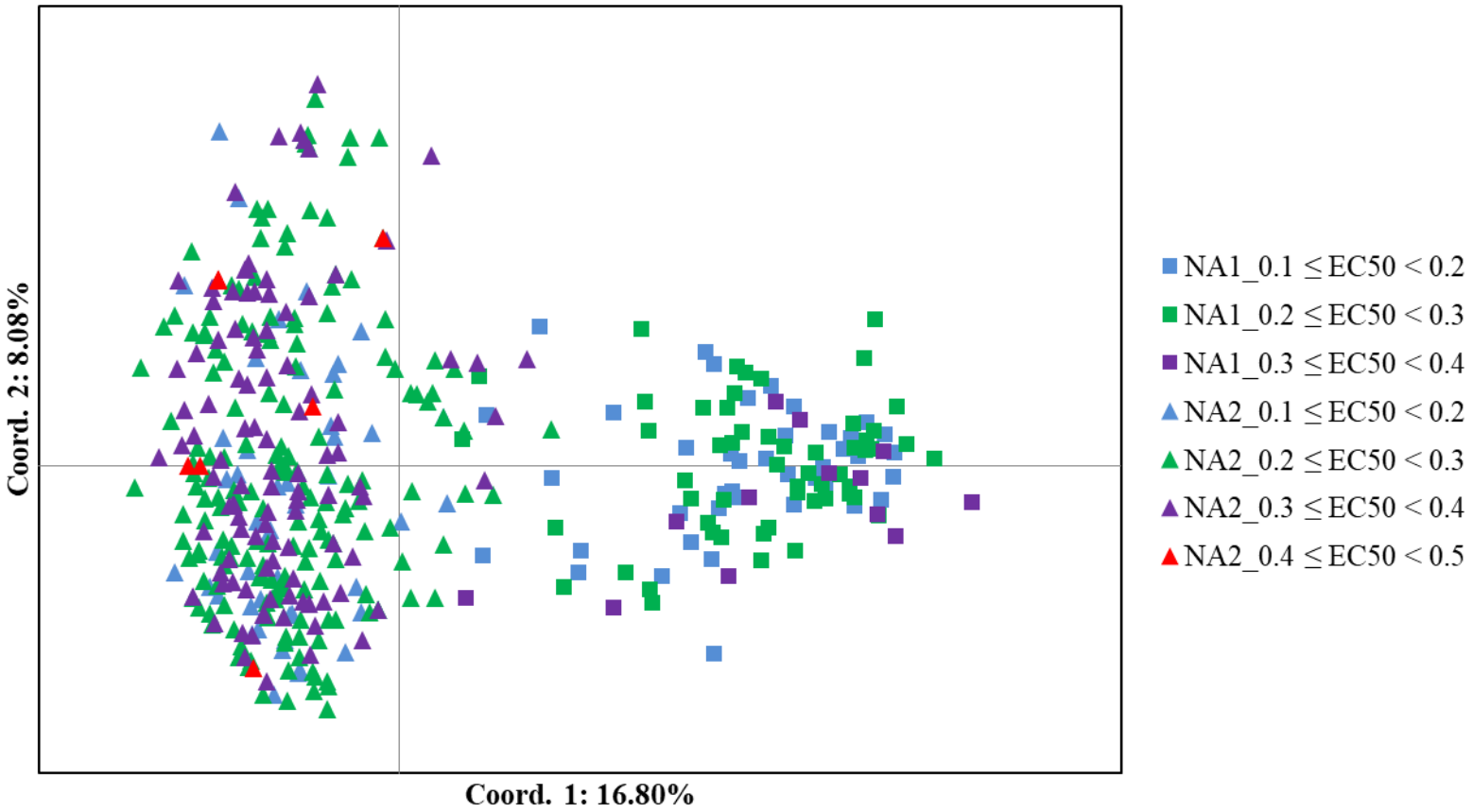

Figure 4-3 Scatter plot of two axes from a Principal Coordinates Analysis of 482 *F. graminearum* isolates, explaining 24.8% of the total observed variation. Shapes denote subpopulation (squares, NA1, triangles NA2). Colours denote isolate EC50 value ranges from the fungicide sensitivity assay.

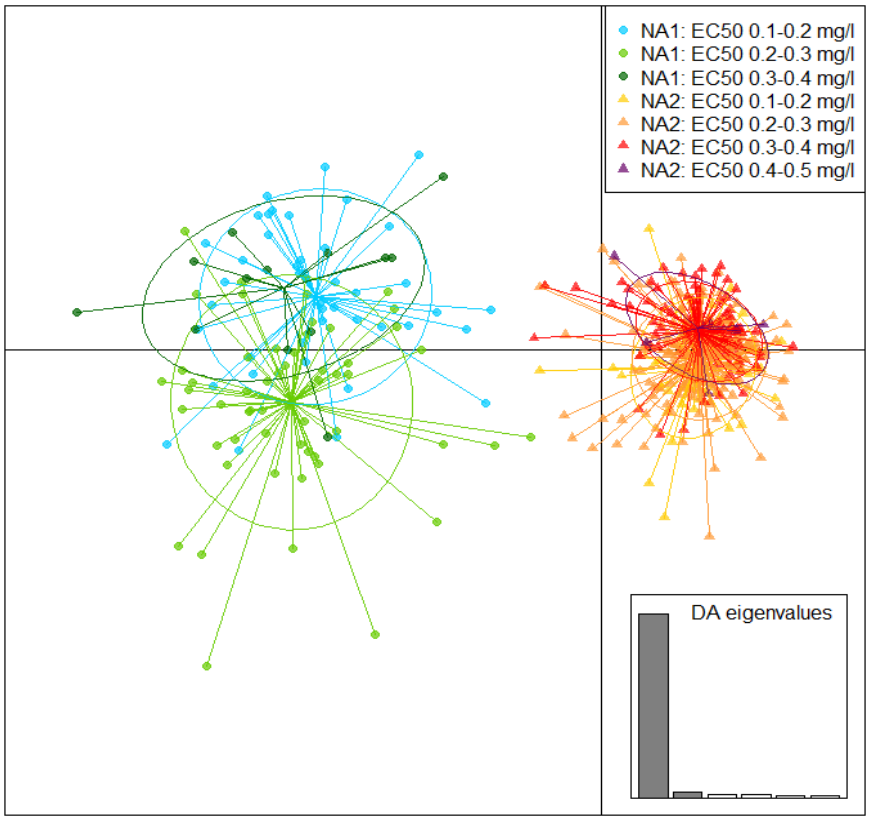

Figure 4-4 Discriminant analysis of principal components for the AFLP data set generated from 452 *Fusarium graminearum* isolates. The number of axes retained for the principal component analysis was 60 and 2 for the discriminant analysis. Seven *a priori* groups were set based on the assigned subpopulation and EC_{50} range from the fungicide sensitivity testing. Points represent individual observations. Lines and shapes and colours represent group membership (subpopulation NA1/NA2 and EC_{50} range within subpopulation) as detailed in the legend (top right). Ellipses represent an analogue of a 67% confidence interval of *a priori* group membership. The insert graph displays the discriminant analysis eigenvalues in relative magnitude with the largest two values in dark grey (bottom right). The first eigenvalue was 714; the second eigenvalue was 23.2.

4.5 Discussion

4.5.1 *In vitro* sensitivity of *F. graminearum* field isolates to Prosaro®

In this study, the *in vitro* sensitivity of isolates to Prosaro® showed a 4-fold range (EC_{50} 0.11 to 0.46 mg L^{-1}). Due to method differences in the resistance assays used in other studies, it is not appropriate to directly compare the sensitivity values in absolute terms from this study to others (Russell, 2008). However, the range in the sensitivity level of the field isolates obtained in this study to the triazole mixture product is much narrower than reported ranges to single triazoles among *F. graminearum* isolates within other collections. For example, the sensitivity to tebuconazole of isolates from New York state ranged from EC_{50} 0.28 to 8.09 mg L^{-1} (Spolti *et al.*, 2014). Isolates from Brazil showed a range of EC_{50} <0.001 to 0.15 mg L^{-1} (Spolti *et al.*, 2012). A range of EC_{50} 0.021 to 1.152 mg L^{-1} was observed in isolates from China (plus one resistant isolate with an EC_{50} of 6.24 mg L^{-1}) (Yin *et al.*, 2009). Sampled field populations in Germany showed high levels of phenotypic variation in sensitivity to propiconazole among isolates with the range of EC_{50} 6 to 56.9 mg L^{-1} in a single field (Talas and Mcdonald, 2015).

It is important to note that a critical difference between the current study and those in the published literature is the use of Prosaro® (a 1:1 mixture of tebuconazole and prothioconazole) rather than a single triazole chemistry. What is unknown without further testing is whether the relatively low levels of variation observed in this study are due to the product tested, or the inherent sensitivity of the field populations sampled. Improvement in the intrinsic potencies of triazoles in *F. graminearum* has occurred over time while gradual shifts in the sensitivity of populations after the introduction of a new chemistry has been observed (Klix *et al.*, (2007).

Tebuconazole was introduced in 1986 and Prothioconazole in 2002 (Morton and Staub, 2008). Therefore, different distributions in the sensitivity of the tested isolates to these chemicals individually and as a mixture would not be unexpected based on both the intrinsic efficacy of tebuconazole *vs*. prothioconazole and sensitivity shifts due to the different levels of exposure of *F. graminearum* populations to these products. Furthermore, studies investigating *in vitro* sensitivity to solo products may highlight greater variation in the sensitivity of the sampled field populations that was in this study confounded by the use of the fungicide mixture.

4.5.2 Effect of fungicide application in the field on the phenotypic variation of *in vitro* fungicide sensitivity of field isolates

ANOVA showed that the application of the fungicide at either timing had no effect on the mean *in vitro* sensitivity of isolates compared to no-fungicide control (Figure 4-2). This provides evidence that fungicide application has not selected for less sensitive isolates in the sampled field populations within a single growing season. This study is limited to two field sites in a single season, and thus does not reflect the full range of agro-environments in Manitoba. Whether the sensitivity of *F. graminearum* populations to triazole fungicides is shifting in Manitoba with respect to a longer temporal period remains to be fully investigated. To the best of the authors knowledge, no previous comparisons of the sensitivity of Canadian *F. graminearum* populations to triazole fungicides has been published and so no inference on shifts in sensitivity overtime can be made without the requisite knowledge of the baseline sensitivity of populations (Russell, 2008). Shifts in the sensitivity of field populations of *F. graminearum* to triazole fungicides have been observed in Germany (Klix *et al.*, 2007) and China (Sun *et al.*, 2014). Tebuconazole resistant isolates have been identified in New York state and China (Yin *et al.*, 2009; Spolti *et*

al., 2014). While these studies have demonstrated the ability of *F. graminearum* populations to adapt to fungicide use, differences in agronomic practices, predominantly exposure levels to triazole fungicides, mean they are by no means indicative of the situation in Manitoba. Further understanding of the sensitivity of historical isolates to triazole fungicides and continued monitoring would provide evidence of whether the fungicide sensitivity of the *F. graminearum* population in Manitoba is shifting, which has implications for the sustainability of disease management practices that include the application of fungicides.

4.5.3 Relating the genotypic and phenotypic diversity in fungicide sensitivity among isolates.

The genetic analysis of isolates using AFLP markers presented in Chapter 3 demonstrated a clear differentiation of the sampled field population into two subpopulations, termed NA1 and NA2. ANOVA showed that there was a significant effect of subpopulation, field study site, and a field study site by subpopulation interaction on the mean EC_{50} of isolates within these groups (Table 4-1). Exploration of the interaction term showed that the mean EC_{50} of isolates assigned to NA1 and NA2 were comparable in the St. Jean Baptiste site. However, in the Brunkild site the mean EC_{50} of isolates assigned to NA2 was higher than isolates assigned to NA1, which in turn was comparable to the two subpopulations in St. Jean Baptiste (Figure 4-2). This interaction suggests that different agronomic factors among the two sites may be contributing to the selection for isolates with lower sensitivity to triazole fungicides within the NA2 subpopulation. This could either be due to agronomic practices in previous seasons that have affected the local field

populations, or the effect of the agronomic practices in the study year on the dynamics of the isolates recovered from the symptomatic spikes.

The primary difference between the sites was the variety used, with a CWRS variety with moderate FHB resistance grown in Brunkild and a CWRS variety with intermediate FHB resistance grown in St Jean Baptiste. This could indicate that the higher resistant variety in combination with fungicide application is selecting for less sensitive isolates within the NA2 subpopulation. A similar trend has been observed in *Mycospharella graminicola* populations with higher tolerance to triazole fungicide in isolates recovered from a resistant host crop (Zhan *et al.*, 2006). The authors of that study hypothesized that traits selected for by the resistant host could also be involved in fungicide tolerance. How this proposed interaction effect impacts the population dynamics on a larger geographical scale, where wheat varieties ranging in FHB resistance levels are grown, is unclear. *In vitro* sensitivity of fifty isolates collected from New York State in 2011 did not differ significantly among 15ADON and 3ADON chemotypes (Spolti et al., 2014). While the study did not confirm the genetic identity of isolates, the findings suggest that across a larger sampling scale, differences in sensitivity are not observed between these subpopulations. Further studies across additional field populations representing more diverse management practices are required to better understand the potential of this hypothesised variety-by-fungicide interaction on the NA2 population.

Linking the AFLP genetic data with the different *in vitro* fungicide sensitivity ranges showed a clear clustering of isolates into the two subpopulations. However, within these subpopulation clusters, there were no distinct groupings of similar genotypes sharing the same sensitivity level

(Figure 4-3). The DAPC analysis further confirmed an absence of genetic differentiation between groupings based on fungicide sensitivity levels (Figure 4-4). However, all isolates with the lowest sensitivity range (0.4 mg $L^{-1} \leq EC_{50} < 0.5$ mg L^{-1}) were found in NA2 in both sites and two pairs of genetically similar isolates were found, possibly indicating the presence of two groups. Due to the low frequency of isolates in this range, any inferences regarding distinct genetic grouping should be treated with caution. As discussed in Section 4.5.1, greater variation in fungicide sensitivity may be observed in solo fungicides, which may give a clearer picture of grouping of genotypes by fungicide sensitivity. Other studies in *Fusarium* species have also observed the absence of genetic clusters of isolates related to *in vitro* sensitivity to triazole fungicides. Linking of genotype data produced by microsatellite-primed polymerase chain reaction to DMI resistant *F. asiaticum* and *F. graminearum* isolates from China showed no clustering based on genotype (Yin *et al.*, 2009). Similarly, no link between genetic diversity inferred from inter simple sequence repeat markers and tebuconazole sensitivity in the closely related species *F. culmorum* was found (Hellin *et al.*, 2017).

Modern fungicides are site-specific in that they target a specific gene in the fungus. Therefore, resistance is often attributed to a limited number of mutations affecting the gene product, such as altered active site of the target enzyme and gene overexpression (Ma and Michailides, 2005). Many of the same mutations can occur in a range of fungal pathogens. For example, the a G143A substitution in the mitochondrial cytochrome b gene has been shown to confer resistance to Quinone outside inhibitor fungicides in powdery mildews (*Blumeria graminis f. sp. Tritici*, *B. graminis f. sp. Hordei*), downy mildew (*Plasmopara viticola*), leaf spots (*Alternaria spp. Mycosphaerella fijiensis*) and rice blast (*Pyricularia grisea*) (Ma and Michailides, 2005). The

occurrence of this single mutation in a broad range of fungal species also means that such single mutations can occur in multiple genotypes within a population of a single species. The control of fungicide sensitivity by a small number of genes could explain why no distinct clustering of *F. graminearum* genotypes by triazole sensitivity was observed in this study and other similar studies.

Resistance to DMI fungicides in fungal cereal pathogens (e.g. *Mycospharella graminicola* and *Blumeria graminis*) has been characterised by alterations in the *CYP51* gene (*e.g.* the Y136F substitution), overexpression of the *CYP51* gene, and overexpression of genes encoding membrane transporters (*e.g.* ATP binding cassette (ABC) transporters) which mediate the efflux of the fungicide molecules from the pathogen cells (Ma and Michailides, 2005; Cools *et al.*, 2013). Often, combinations of these mechanisms can be found to confer resistance in the fungal organism, demonstrating a polygenic nature of DMI resistance (Cools *et al.*, 2013). The mechanism of triazole resistance in *F. graminearum* remains unclear. *Fusarium graminearum*, has three *CYP51* paralogs and knock-out mutants have demonstrated their role in triazole sensitivity (Liu *et al.*, 2011; Fan *et al.*, 2013). ABC transporters have also been implicated in determining sensitivity to triazoles in knock-out mutagenesis studies (Ammar *et al.*, 2013; Qi *et al.*, 2018). While these support similar modes of resistance to other pathogens, studies on resistant field isolates have pointed to alternative modes of resistance yet to be determined (Yin *et al.*, 2009; Fan *et al.*, 2013; Talas and Mcdonald, 2015). A unique approach by Talas *et al.*, (2016) utilised a genome-wide association study on 220 *F. graminearum* field isolates from Germany and identified 74 quantitative trait nucleotides and 51 genes associated with *in vitro* propiconazole sensitivity. While the roles of these candidate genes are yet to be validated, the

study supports a polygenic nature of triazole fungicide sensitivity in *F. graminearum*. Investigation into the variation in these putative genes in the field isolates recovered in this study could provide valuable insight into the standing variation present in the field populations and therefore, potential for adaption to fungicide use. It would also be of interest to compare the variation in these genes among the two subpopulations, NA1 and NA2, in Canadian isolates given the differences in sensitivity levels observed in this study. Genomic analysis of U.S. and Canadian isolates found that one of the genes identified by Talas *et al.*, (2016), a transcriptional regulator of fungicide resistance had signatures of selection in NA1 isolates, but not NA2 isolates, demonstrating differences between these subpopulations in a proposed trait for fungicide sensitivity (Kelly and Ward, 2018).

4.6 Conclusions

The results of this study demonstrated a low level of variation among *F. graminearum* field isolates in the *in vitro* sensitivity to the prothioconazole and tebuconazole mixture product Prosaro®. There was no significant evidence that triazole fungicide application acted as a selective force on the field populations within the single field season, as no differences in sensitivity levels among isolates from different field treatments were observed. Furthermore, no distinct genetic groups with a unique sensitivity range were detected. These findings suggest that triazole fungicide application in the field for FHB control is not rapidly selecting for isolates with reduced sensitivity. However, this study was limited to a single growing season, with a single generation in the pathogen population, so longer term studies would be required to fully understand the selection imposed by triazole fungicide application.

Differences in the *in vitro* fungicide sensitivity observed between subpopulations at a single site may indicate that other management practices could be driving selection for a population with a reduced sensitivity level to triazole fungicides. Further investigation with a larger number of field populations representing a wider geographical range and management practices, including other triazole fungicides used for the control of FHB is required to fully explore this effect. The evidence of a shift of sensitivity levels across the two subpopulations in the Brunkild trial site warrants the continued monitoring of fungicide sensitivity levels of populations, to ensure the sustainability of this tool as part of an integrated pest management strategy for FHB control.

5 GENERAL DISCUSSION

The aim of this research project as a whole was to assess the effect of a typical foliar triazole fungicide protocol on *F. graminearum* field populations recovered from naturally infected symptomatic wheat spikes in Manitoba. To reach this objective, two studies were conducted as reported in Chapter 3 and Chapter 4. The first study used AFLP markers to genotype *F. graminearum* isolates recovered from symptomatic wheat spikes sampled from fungicide treated and untreated field plots. Marker data was then analysed to investigate the effect in the fungicide treatment on the genetic structure of the sampled field populations. The second study assessed the phenotypic variation of the *F. graminearum* isolates to *in vitro* fungicide sensitivity to the same triazole mixture product applied in the field trials. Considering the two research studies together, a clear understanding of the selective pressure exerted by fungicide application in a single growing season in terms of both the field population structure and the phenotypic fungicide sensitivity of isolates was made.

Across two CWRS wheat trial sites, fungicide application at two different timings did not have a significant effect on the population genetic structure of isolates. DAPC analysis suggested some minor genetic differentiation between isolates recovered from the different field fungicide treatments in the NA1 subpopulation. Overall, a narrow range in the *in vitro* sensitivity of isolates was observed, with no significant difference in the mean EC_{50} of isolates recovered from the different field fungicide treatments. While variation in sensitivity of recovered isolates to the fungicide product was observed, no clustering of the individuals within defined sensitivity ranges

based on genotype was identified. These data demonstrate that the fungicide application has not selected for specific genotypes within a single growing season. However, as discussed, the use of a mixture product throughout this study could be confounding effects that would be observed with a solo product, due to different efficacies of each individual active ingredient. Understanding the responses between dual and single chemistry products should also be assessed to investigate the possibility of selection imposed by a single product.

Considering the weight of evidence from both experimental studies, this research has provided no evidence that a dramatic shift in fungicide sensitivity will occur as a result of using a recommended product. As such, when considered at a high level, the finding of this research would support the continued use of fungicide as a key component of IPM. This conclusion is, perhaps, an expected result given that FHB is monocyclic disease with only one generation per season, combined with the evidence that suggests *F. graminearum* sensitivity to triazole fungicides, like other fungal pathogens, is polygenic. Furthermore, *F. graminearum* has both a parasitic and saprophytic life cycle, combined with pathogenicity on multiple crop and non-crop hosts, of which some will not be exposed to triazole fungicides. This could also contribute to a lower risk of fungicide sensitivity shifts occurring due to balancing selection between these different environments. However, evidence of population sensitivity shifts and identification of resistant isolates in other countries highlight the importance of continued monitoring of sensitivity levels in *F. graminearum* populations in Manitoba.

The AFLP method provided a detailed genetic characterisation of the field populations sampled. As with previous studies, high genotypic diversity and the presence of two subpopulations,

associated with trichothecene genotype, were observed. In comparison to the most recent study on a collection of NA1 and NA2 isolates from Manitoba recovered from 2005 to 2007, this study has indicated a further weakening of the correlation between trichothecene genotype and subpopulation (15ADON/NA1 and 3ADON/NA2). These data show the importance of validating PCR-based trichothecene genotyping with trichothecene chemical confirmation for studies intending to interpret results based on chemotype. This study has also demonstrated a stabilisation of trichothecene genotype frequencies in Manitoba, with 15ADON and 3ADON frequencies similar to those observed since 2004. As discussed, there is speculation that the increased corn acreage in Manitoba could be supporting the 15ADON genotype / NA1 genetic background. Investigation into the genetic population dynamics of *F. graminearum* isolates recovered from corn would be required to support this hypothesis.

In this study the two trial sites that were sampled had similar agronomic features: they both had soybean as the previous year's crop, the same triazole fungicide product was applied, and they were geographically close, and therefore, experienced a similar climate. There was also low genetic differentiation between the *F. graminearum* populations sampled from each site. While the single identifiable difference between the two sites, in this case CWRS wheat variety, was not a primary factor intended for investigation, observed differences across sites in some population characteristics and fungicide sensitivities could be attributable to variety and a variety by fungicide application interaction. The moderately resistant variety grown in Brunkild was associated with both reduced admixtures (and therefore reduced recombination between NA1 and NA2 subpopulations) and reduced gene diversity within the NA2 subpopulation, compared to the intermediate resistant variety grown in St. Jean Baptiste. The *in vitro* sensitivity of isolates

also showed a site by subpopulation interaction. Both NA1 and NA2 from St. Jean Baptiste and NA1 from Brunkild had similar average EC_{50} values, whereas the average EC_{50} for isolates assigned to NA2 from Brunkild was higher. This could indicate that the moderately resistant variety is selecting for a lower sensitivity population in NA2 and this selection pressure is resulting in reduced genetic diversity. Alternatively, previous agronomic practices may have influenced the local *F. graminearum* populations, which was then observed in the isolates recovered from the symptomatic spikes in this study.

Overall, this study suggests the application of a triazole fungicides for the control of FHB does not have a significant effect on the of genetic and phenotypic population dynamics of *F. graminearum* within a single growing season. Site-specific triazole fungicide sensitivity levels within the predominant NA2 subpopulation may be indicative of an impact of current management strategies, in particular wheat resistance level, on local field population dynamics. Longer-term studies across a greater number of sites, representative of a wider range of agronomic environments and disease levels are required to better understand the role of fungicide use and other management practices on the population dynamics of *F. graminearum* that may in turn affect the efficacy of fungicides. Furthermore, continued monitoring of triazole fungicide sensitivity levels in the *F. graminearum* population in Manitoba is warranted to ensure the sustainability of this technology.

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7 APPENDICES

Appendix 7.1 Media Recipes

0.5 X / 1 X Potato dextrose agar (PDA) and PDA plus Streptomycin

20 g (0.5 X), 39 g (1 X), PDA (Difco Laboratories, MD)

1 L tap water

1 ml 100 ppm Streptomycin sulphate salt (Sigma-Aldrich, MO, US)

Dissolve PDA in tap water and autoclave at 121°C for 45 minutes.

For PDA plus Streptomycin, prepare a stock of 100 ppm streptomycin sulphate by dissolving 200 mg of streptomycin sulphate in 2 ml of distilled water and filter sterilise through a 0.2 µm syringe PES filter (25mm, Whatman plc, GE Healthcare, IL, US). Allow PDA to cool to ~55°C and add 1ml of the streptomycin stock.

Synthetic Nutrient Agar (SNA) (recipe adapted from Nirenberg, (1977))

1.0 g KH_2PO_4

0.5 g $MgSO_4 \cdot 7H_2O$

0.5 g KCl

0.2 g Glucose

0.2 g Saccharose

1.0 g KNO_3

6.0 ml 1 M NaOH

17 g granulated agar (Difco Laboratories, MI, US)

1 L tap water

1 x 1 cm sterile filter paper

Dissolve dry ingredients in 1 L tap water, omitting KNO_3 and agar. Dissolve KNO_3 into the solution followed by the agar and NaOH. Autoclave at 121°C for 45 minutes. Once agar has been poured into petri-dish, add 1 square of 1 x 1 cm sterile filter paper.

Synthetic Nutrient Broth (SNB) (recipe adapted from Talas and Mcdonald, (2015))

2g KH_2PO_4

2g KNO_3

1g $MgSO_4 \cdot 7H_2O$

1g KCl

2g Sucrose

1L tap water

Dissolve dry ingredients in 1 L tap water, omitting KNO_3 Dissolve KNO_3 into the solution. Autoclave at 121°C for 45 minutes.

Czapek-Dox Broth (CDB) (recipe adapted from Thom and Church, (1926))

3g NaNO (Sodium nitrate)

1g K2HPO4 (Dipotassium phosphate)

0.5g $MgSO_4$ (Magnesium sulfate)

0.5g KCl (Potassium chloride, BDH Chemicals, Toronto, CA)

0.01g $FeSO_4$ (Ferrous sulfate)

30g Sucrose (Sigma-Aldrich, St. Louis, MO, USA)

1L tap water

Dissolve dry ingredients in 1L tap water. Autoclave at 121°C for 45 minutes.

Appendix 7.2 Fungicide Sensitivity Bioassay Method Development

7.2.1 Introduction

A high throughput microtiter plate assay was developed to evaluate the *in vitro* sensitivity of 453 *F. graminearum* isolates to Prosaro®, a commercial 1:1 mixture product of tebuconazole and prothioconazole. Other commonly used methods to assess *in vitro* sensitivity to fungicides include spore germination assessments and radial growth on agar plate assessment (Klix *et al.*, 2007; Becher *et al.*, 2010; Spolti *et al.*, 2014). Neither of these methodologies were considered appropriate for the objective of this study as both would have been too resource intensive for the large number of isolates. For example, with a spore germination or radial mycelial growth assay, using six fungicide rates, at two replicates per test, repeated independently in two experiments equates to 10,872 plates. Therefore, a high throughput assay in the form of 96-well microtiter plate with a spectrophotometric assessment for easy data management and calculation of concentration of tebuconazole and prothioconazole corresponding to 50% growth inhibition relative to the maximum and minimum growth (EC_{50}) from a dose response curve for each isolate was developed.

Fungicide concentrations were selected based on the criteria that they cover the sensitivity range of the sampled population. For the analysis of the response of each isolate using a dose response curve, the fungicide concentration range that gave maximum response *i.e*. a rate that gives total control (no growth), and a rate that gave the minimum response, *i.e.* same growth as no fungicide control, and sufficient doses within these extremes was required to allow an accurate assessment of the shape of the dose response curve and EC_{50} estimation. The assay also needed to be

reproducible to enable assessment of the variation inherent in the fungicide sensitivity of the sampled populations and reduce the variability due to the bioassay. The requirement for a spectrophotometric assessment meant that mycelial growth within plate wells needed to be uniform to reduce variability within the test.

7.2.2 Materials and Methods

Frozen aliquots of conidial suspensions were prepared as described in Section 4.3.1. Aliquots were prepared at 2.5 x 10^5 conidia / ml for initial experiments and 2.5 x 10^4 conidia / ml for the final assay validation. All method development assays were conducted as described in Sections 4.3.2 except for media and assessment times where indicated. Assay fungicide concentrations of 0.059, 0.23, 0.94, 3.75, 15, 60 and 240 mg L^{-1} were used in the first development assay in Synthetic Nutrient Broth (SNB); all subsequent development tests used concentrations of 0.0077, 0.384, 0.192, 0.96, 4.8, 24 and 120 mg L^{-1}. Czapek Dox Broth (CDB) and (SNB) were prepared as in Appendix 7.1. Dose response curve fitting and EC_{50} estimation was performed as described in Section 4.3.3 using three technical replicates. The assay was validated by performing the final chosen method on twelve randomly selected isolates. Each isolate was tested across two separate plates with three replications per fungicide dose / plate. The experiment was independently replicated twice.

7.2.3 Results and Discussion

Fusarium graminearum grew poorly in SNB with weak and non-homogenous mycelial growth. As a result of the weak growth, the assessment was performed at five days. The non-homogenous growth resulted in a poor fit of the data to a dose response curve (Figure 7-1),

resulting in large standard errors associated with the EC_{50} estimations of the four isolates tested (Table 7-1). The fungicide concentrations used resulted in a plateau in the maximum response with the highest five concentrations in all four isolates. For one isolate (SJ4LS3) the lowest concentration of 0.059 mg L^{-1} did not give the same response as the no fungicide control, indicating that the minimum response was outside of the concentration range tested (Figure 7-1). Therefore fungicide concentrations were adjusted to 0.0077, 0.384, 0.192, 0.96, 4.8, 24 and 120 mg L^{-1} for subsequent development assays in order to cover fungicide concentrations that give a minimal response, reduce the number of concentrations that give a maximum response, and increase the number of fungicide concentrations between these parameters. CDB produced higher levels of growth compared to SNB, and the data showed an improved fit to the dose response curve (Figure 7-2). Although the observed mycelial growth was more homogenous than in SNB, there was still clumping that created variation among technical replicates when assessed with the plate reader. This resulted in large error in the EC_{50} estimation at both four days after treatment (DAT) (standard error (SE) range 0.045 – 0.456) and five DAT (SE range 0.063 to 1.059) (Table 7-1).

Initial experiments using PDB with a range of conidia concentrations showed insufficient growth at 2 DAT (Figure 7-3). At conidia concentrations of 1.25×10^4 and 2.5×10^4, mycelial growth was observed outside of individual wells by 3 DAT. These conidia concentrations were therefore deemed unsuitable due to potential for cross-contamination in plates. Data had an improved fit to the dose response curve at lower conidia concentrations of 1.25×10^3 and 2.5×10^3 at 3 DAT with a low amount of error in the EC_{50} estimation (SE 0.017 and 0.042 respectively) (Figure 7-3, Table 7-1). A further investigation was conducted for a range of

conidia concentrations between 5 x 10^2 and 1 x 10^4 at 3 DAT and 4 DAT assessments using three replicate plates per test. All conidia concentrations in this range showed a good fit of the data to the dose response curve (Figure 7-4, Figure 7-5). The standard error for EC_{50} estimates was lower for assessments at 3 DAT than 4 DAT across all conidia concentrations tested (Table 7-1). This can be attributed to an observed reduction in the level of homogenised mycelial growth and pigmentation observed at 4 DAT compared to 3 DAT. The highest conidia concentration of 1 x 10^4 exhibited mycelial growth outside of individual wells by 3 DAT and was therefore deemed too high. As the standard error for EC_{50} estimates were similar among the other conidia concentrations, a final conidia concentration of 2.5 x 10^3 that was within this range was chosen for further assay development.

In summary, poor growth in SNB and CDB led to the selection of PDB as growth media. Test duration at 2 DAT in PDB gave weak growth at all conidia concentrations. Within the range of conidia concentrations tested, 3 DAT gave a lower standard error of EC_{50} estimation than 4 DAT. This improved precision in EC_{50} estimation at 3 DAT over 4 DAT was due to more uniform growth at the earlier assessment, which is better suited for optical density measurements. The range of fungicide concentrations used was sufficient to display a sigmoidal dose response curve with clear minimum and maximum responses and sufficient concentrations in between to describe the shape of the curve. This preliminary method development resulted in a final assay design using PDB, a conidia concentration of 2.5 x 10^3 and a 3 DAT assessment. This allowed testing of up to 72 isolates per day in three tests per week by a single researcher.

The final assay method was assessed for reproducibility using twelve randomly selected isolates. The Coefficient of Variation (CV) within plates of the same test ranged from 1.4 % to 31.6 % with an average of 12.9%. The CV across tests ranged from 5.0 % to 32.3% with an average of 19.8 %. The final method was therefore deemed suitable for use across the multiple tests required to screen all 453 isolates.

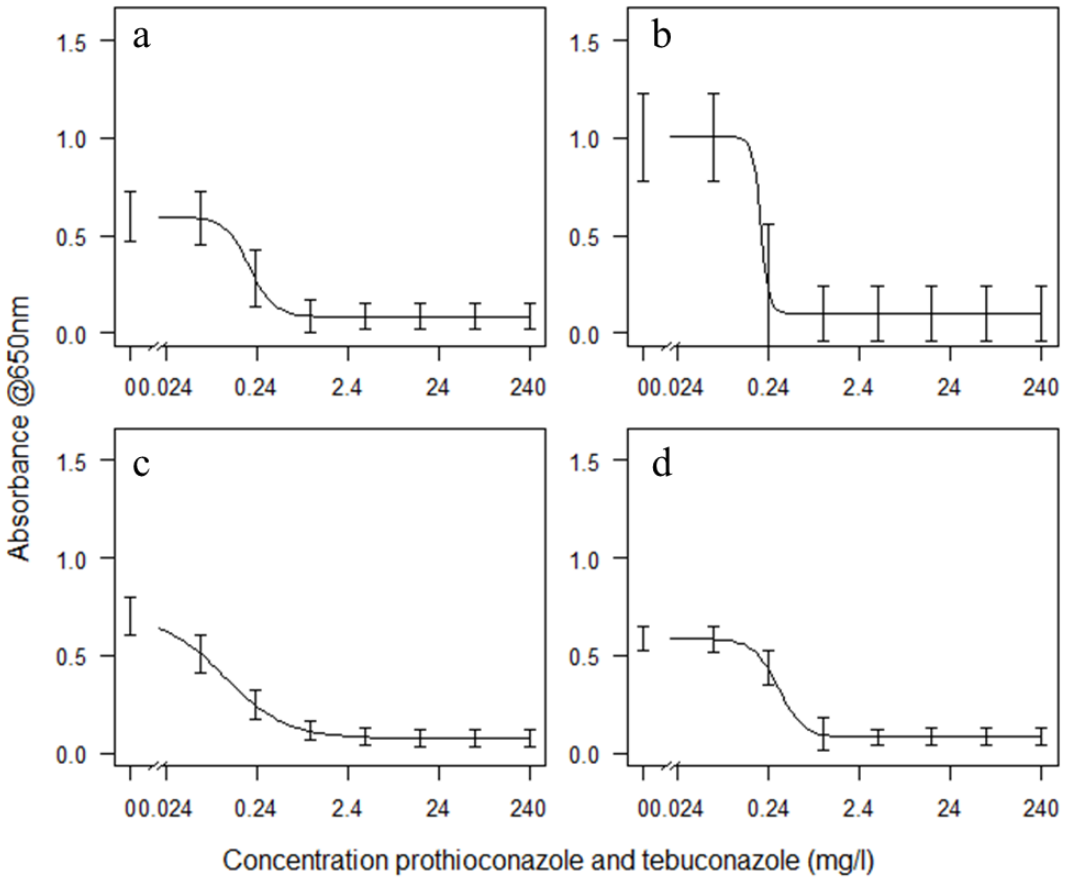

Figure 7-1 Dose-response curves of four *F. graminearum* isolates grown in synthetic nutrient broth (SNB) with a 1:1 mixture of prothioconazole and tebuconazole. Plates were assessed 4 DAT. All conidia concentrations were at 2.5 x 10^4. a = isolate SJ4LS2, b = isolate SJ4LS3, c = isolate SJ4LS4, d = isolate SJ4LS6. Bars are 95% confidence intervals.

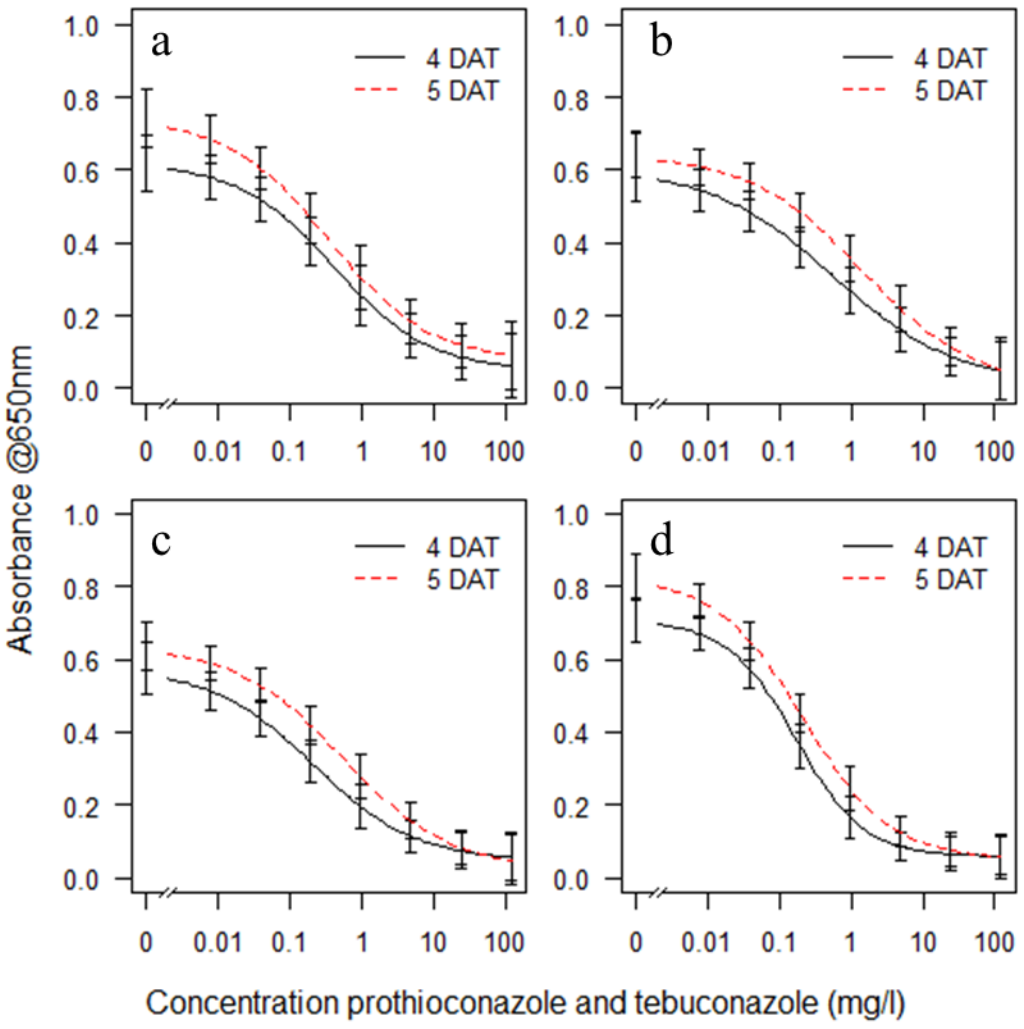

Figure 7-2 Dose-response curves of four *F. graminearum* isolates in grown in Czapeks Dox Broth (CDB) with a 1:1 mixture of prothioconazole and tebuconazole. Plates were assessed 4 DAT and 5 DAT. All conidia concentrations were at 2.5 x 10^4. a = isolate SJ4LS2, b = isolate SJ4LS3, c = isolate SJ4LS4, d = isolate SJ4LS6. Bars are 95% confidence intervals.

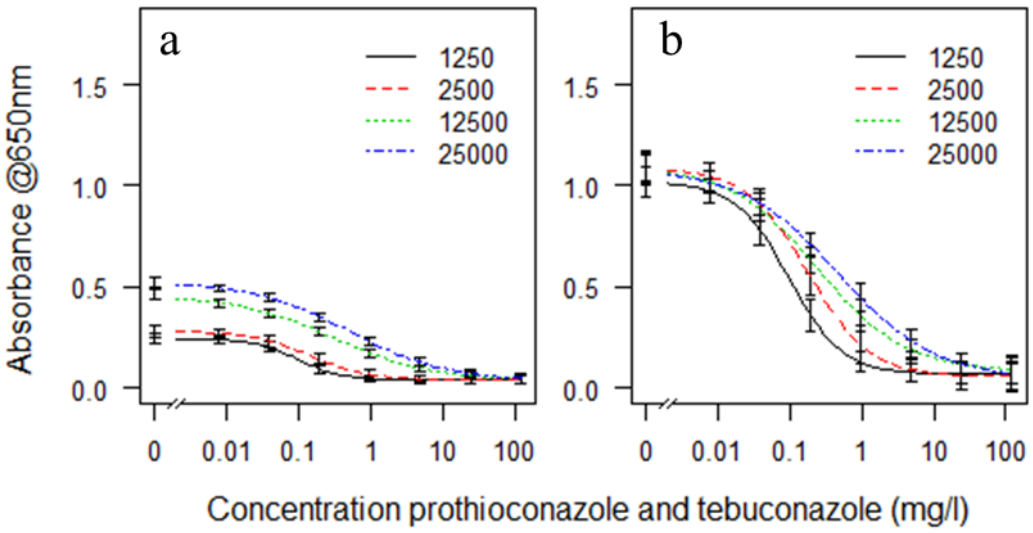

Figure 7-3 Dose-response curves for *F. graminearum* isolate SJ4LS4 in grown in PDB with a 1:1 mixture of prothioconazole and tebuconazole at four conidia concentrations (1.25×10^3 (1250), 2.5×10^3 (2500), 1.25×10^4 (12500), and 2.5×10^4 (25000)). Plates were assessed 2 DAT (a) and 3 DAT (b). Bars are 95% confidence intervals.

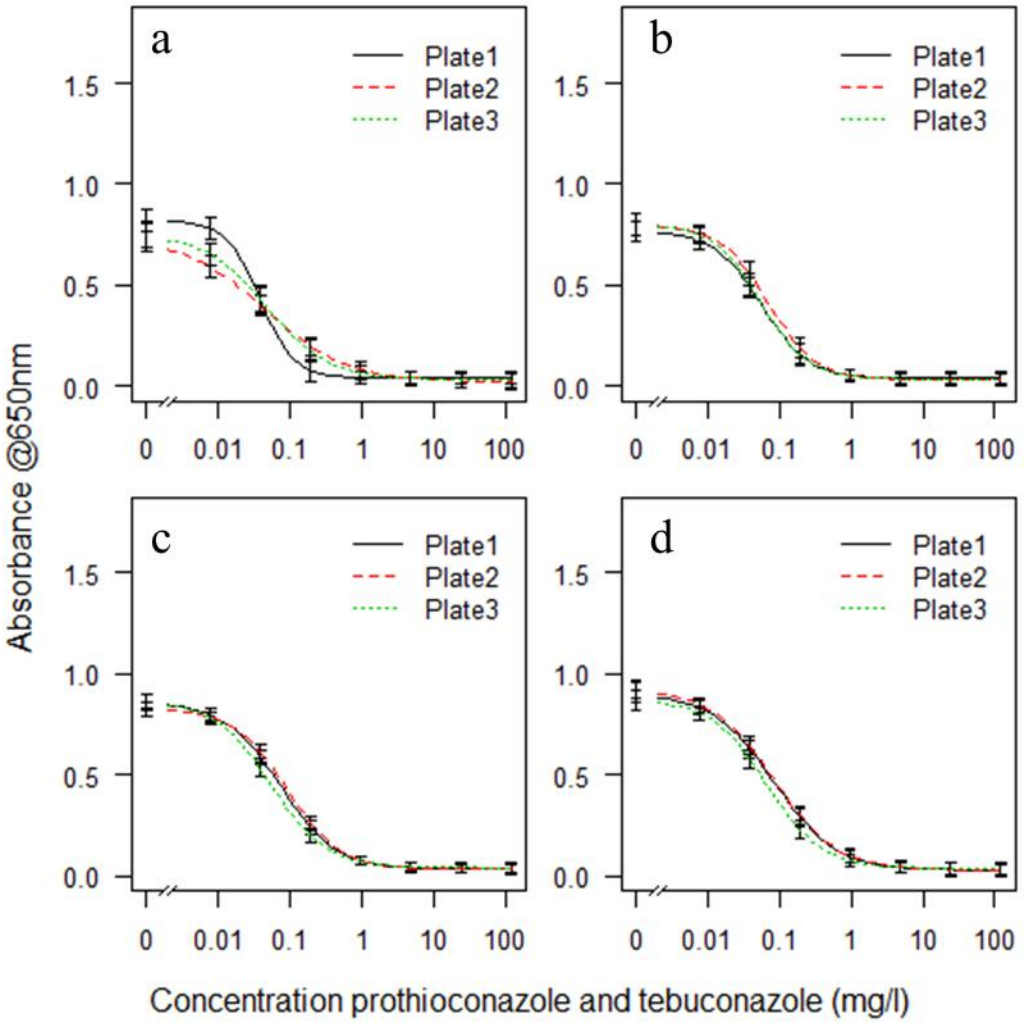

Figure 7-4 Dose-response curves for *F. graminearum* isolate SJ1US15 grown in PDB with a 1:1 mixture of prothioconazole and tebuconazole at four conidia concentrations (a = 5 x 10^2, b = 1 x 10^3, c = 5 x 10^3, d = 1 x10^4) replicated across three plates. Plates were assessed 3 DAT. Bars are 95% confidence intervals.

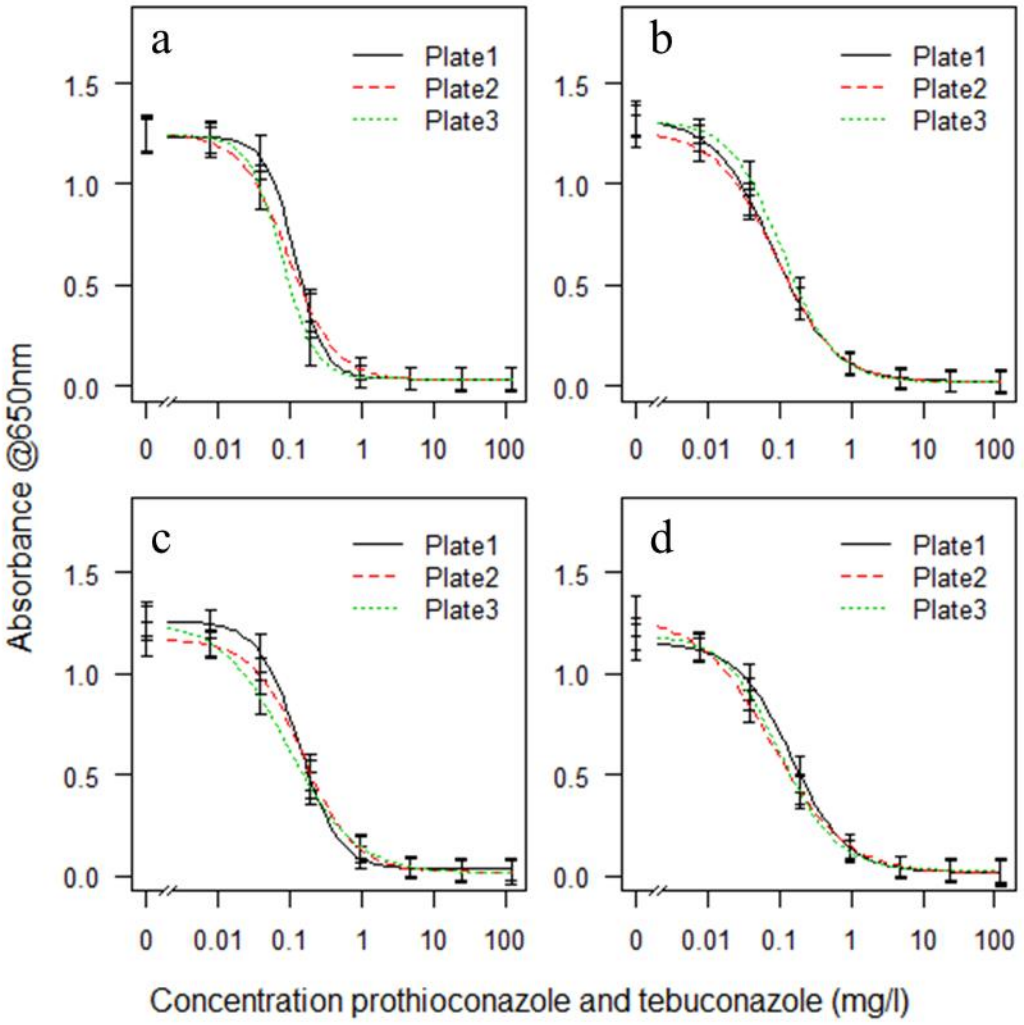

Figure 7-5 Dose-response curves for *F. graminearum* isolate SJ1US15 grown in PDB with a 1:1 mixture of prothioconazole and tebuconazole at four conidia concentrations (a = 5 x 10^2, b = 1 x 10^3, c = 5 x 10^3, d = 1 x10^4) replicated across three plates. Plates were assessed 4 DAT. Bars are 95% confidence intervals.

Table 7-1 Summary of method development experiments

Isolate	Media[a]	Plate replicate	Conidia rate (conidia / ml)	DAT[b]	EC_{50}[c]	SE[d]	2.5% CI[e]	97.5% CI[f]
SJ4LS2	SNB	1	2.50E+04	5	0.203	0.068	0.062	0.344
SJ4LS3	SNB	1	2.50E+04	5	0.197	0.231	-0.283	0.678
SJ4LS4	SNB	1	2.50E+04	5	0.108	0.029	0.049	0.168
SJ4LS6	SNB	1	2.50E+04	5	0.309	0.078	0.147	0.471
SJ4LS2	CDB	1	2.50E+04	4	0.407	0.267	-0.133	0.946
SJ4LS2	CDB	1	2.50E+04	5	0.341	0.224	-0.113	0.794
SJ4LS3	CDB	1	2.50E+04	4	0.555	0.456	0.367	1.477
SJ4LS3	CDB	1	2.50E+04	5	1.424	1.059	-0.717	3.565
SJ4LS4	CDB	1	2.50E+04	4	0.211	0.122	-0.034	0.457
SJ4LS4	CDB	1	2.50E+04	5	0.534	0.293	-0.058	1.126
SJ4LS6	CDB	1	2.50E+04	4	0.168	0.045	0.077	0.259
SJ4LS6	CDB	1	2.50E+04	5	0.212	0.063	0.762	0.889
SJ4LS4	PDB	1	1.25E+03	2	0.097	0.023	0.050	0.143
SJ4LS4	PDB	1	2.50E+03	2	0.142	0.043	0.057	0.228
SJ4LS4	PDB	1	1.25E+04	2	0.290	0.070	0.150	0.431
SJ4LS4	PDB	1	2.50E+04	2	0.497	0.108	0.282	0.711
SJ4LS4	PDB	1	1.25E+03	3	0.097	0.017	0.063	0.131
SJ4LS4	PDB	1	2.50E+03	3	0.180	0.042	0.097	0.263
SJ4LS4	PDB	1	1.25E+04	3	0.245	0.071	0.104	0.385

SJ4LS4	PDB	1	2.50E+04	3	0.478	0.153	0.173	0.782
SJ1US15	PDB	1	5.00E+02	3	0.038	0.004	0.030	0.046
SJ1US15	PDB	2	5.00E+02	3	0.045	0.011	0.022	0.068
SJ1US15	PDB	3	5.00E+02	3	0.047	0.010	0.028	0.066
SJ1US15	PDB	1	1.00E+03	3	0.056	0.008	0.041	0.071
SJ1US15	PDB	2	1.00E+03	3	0.069	0.009	0.051	0.088
SJ1US15	PDB	3	1.00E+03	3	0.055	0.007	0.041	0.069
SJ1US15	PDB	1	5.00E+03	3	0.073	0.007	0.058	0.088
SJ1US15	PDB	2	5.00E+03	3	0.089	0.009	0.071	0.107
SJ1US15	PDB	3	5.00E+03	3	0.054	0.005	0.043	0.065
SJ1US15	PDB	1	1.00E+04	3	0.082	0.011	0.060	0.105
SJ1US15	PDB	2	1.00E+04	3	0.084	0.011	0.062	0.106
SJ1US15	PDB	3	1.00E+04	3	0.066	0.008	0.049	0.083
SJ1US15	PDB	1	5.00E+02	4	0.118	0.017	0.084	0.151
SJ1US15	PDB	2	5.00E+02	4	0.094	0.016	0.063	0.126
SJ1US15	PDB	3	5.00E+02	4	0.076	0.011	0.055	0.097
SJ1US15	PDB	1	1.00E+03	4	0.083	0.013	0.058	0.108
SJ1US15	PDB	2	1.00E+03	4	0.091	0.015	0.061	0.120
SJ1US15	PDB	3	1.00E+03	4	0.110	0.015	0.079	0.140
SJ1US15	PDB	1	5.00E+03	4	0.133	0.018	0.098	0.169
SJ1US15	PDB	2	5.00E+03	4	0.153	0.026	0.101	0.206
SJ1US15	PDB	3	5.00E+03	4	0.093	0.018	0.057	0.129

SJ1US15	PDB	1	1.00E+04	4	0.148	0.027	0.093	0.203
SJ1US15	PDB	2	1.00E+04	4	0.080	0.016	0.047	0.112
SJ1US15	PDB	3	1.00E+04	4	0.101	0.018	0.066	0.138

[a] CDB = Czapeks Dox Broth, SNB = Synthetic Nutrient Broth, PDB = Potato Dextrose Broth
[b] Days after treatment.
[c] Concentration of tebuconazole and prothioconazole corresponding to 50% growth inhibition relative to the maximum and minimum growth.
[d,e,f] Standard error, 2.5% confidence interval and 97.5% confidence interval for estimated EC_{50} values respectively.

Table 7-2 Method validation summary of estimated EC_{50} values and %CV across plates within tests and %CV across tests for twelve *Fusarium graminearum* isolates.

Isolate	**Test**	**Plate**	**EC_{50} (mg / L)[a]**	**% CV[b] across plates within tests**	**%CV across plates and tests**
SJ2LS19	1	1	0.17	1.4	5.0
		2	0.17		
	2	1	0.17	7.4	
		2	0.15		
SJ2RS8	1	1	0.11	7.2	26.2
		2	0.10		
	2	1	0.18	26.0	
		2	0.12		
SJ3LS12	1	1	0.11	24.8	32.3
		2	0.15		
	2	1	0.23	25.2	
		2	0.16		
SJ3RS1.2	1	1	0.16	6.3	13.3
		2	0.15		
	2	1	0.20	17.6	
		2	0.15		
SJ3RS12	1	1	0.12	11.7	27.4
		2	0.14		
	2	1	0.22	2.1	
		2	0.21		
SJ3RS2	1	1	0.19	11.6	15.5
		2	0.16		
	2	1	0.17	20.8	

		2	0.23		
SJ3RS8	1	1	0.15	6.0	21.9
		2	0.17		
	2	1	0.14	31.6	
		2	0.23		
SJ3RS9	1	1	0.11	10.4	22.2
		2	0.13		
	2	1	0.18	3.2	
		2	0.18		
SJ3US15	1	1	0.15	6.8	23.8
		2	0.16		
	2	1	0.24	30.9	
		2	0.15		
SJ3US16	1	1	0.16	3.6	15.8
		2	0.15		
	2	1	0.22	11.8	
		2	0.18		
SJ3US19	1	1	0.20	16.5	12.8
		2	0.16		
	2	1	0.15	6.7	
		2	0.16		
SJ3US20	1	1	0.17	2.1	20.7
		2	0.16		
	2	1	0.25	18.0	
		2	0.19		
Overall				12.9	19.8

[a]Calculated from three technical replicates per plate
[b]Coefficient of variation = (standard deviation / mean) * 100

7.2.4 Conclusion

A high-throughput 96-well microtiter plate based assay was developed for the assessment of *in vitro* sensitivity of *F. graminearum* to a mixed product of tebuconazole and prothioconazole. Optimisation of media, conidia rate and assessment timing was performed to ensure a satisfactory level of reproducibility of EC_{50} estimation for individual isolates across multiple tests as demonstrated with the average %CV of 19.8% across tests in the method validation

(Table 7-2). The final method facilitated the screening of the 453 *F. graminearum* isolates described in Chapter 4 in an efficient and manageable approach.

Appendix 7.3 Analysis of variance diagnostic plots

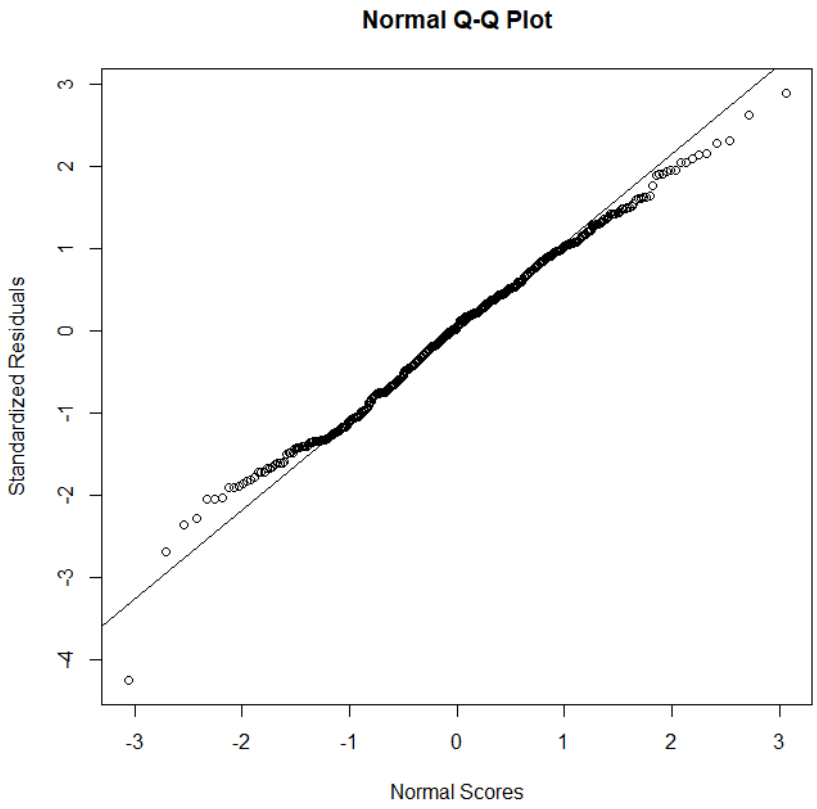

Figure 7-6 Normal probability plot of standardized residuals from the fit of the ANOVA relating EC_{50} to a 1:1 mixture of prothioconazole and tebuconazole to site, subpopulation and fungicide treatment.

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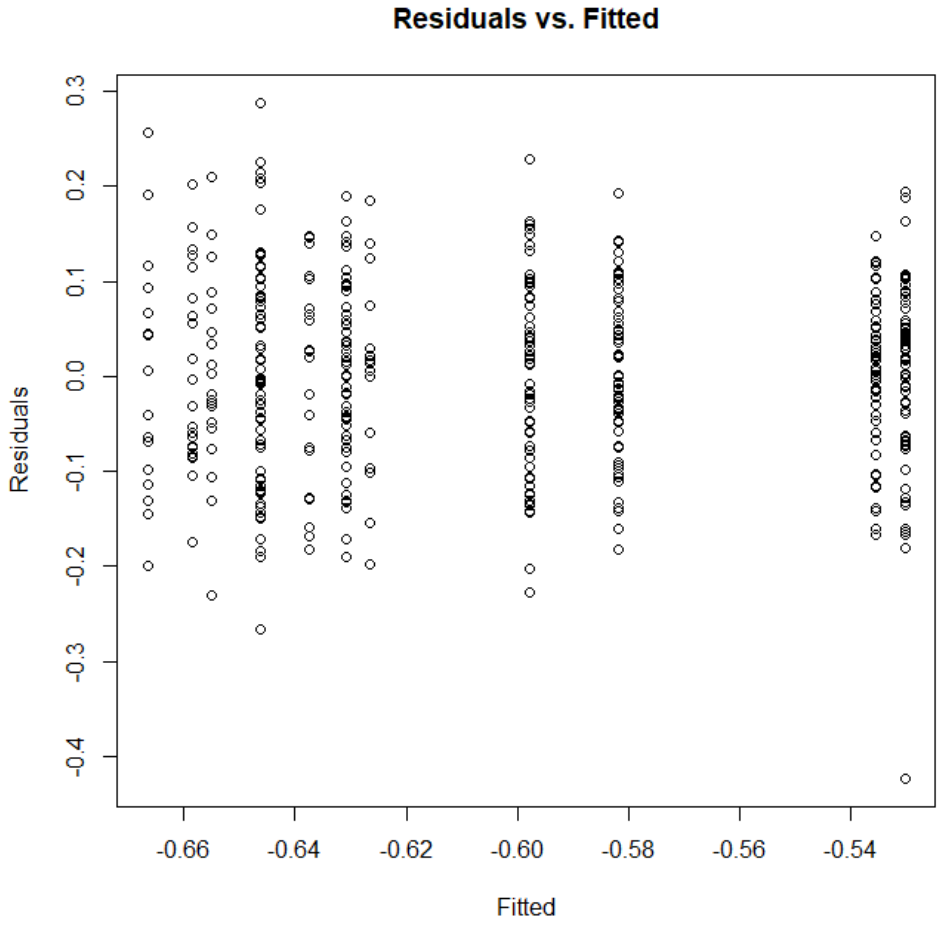

Figure 7-7 Plot of residuals from the fit of the ANOVA relating EC_{50} to a 1:1 mixture of prothioconazole and tebuconazole to site, subpopulation and fungicide treatment.

Appendix 7.4 List of abbreviations

15ADON 15-acetyldeoxynivalenol

3ADON 3-acetyldeoxynivalenol

AFLP Amplified fragment length polymorphism

AMOVA Analysis of molecular variance

ANOVA Analysis of variance

CDB Czapek Dox broth

CWRS Canada western red spring

DAPC Discriminant analysis of principal components

DMI Demethylation inhibitor

DON Deoxynivalenol

ELISA Enzyme-linked immunosorbent assay

FDK Fusarium damaged kernels

FHB Fusarium head blight

FUS DMG Fusarium damage

IPM Integrated pest management

MLG Multilocus genotypes

NIV Nivalenol

PCoA Principle coordinates analysis

PCR Polymerase chain reaction

PDA Potato dextrose agar

PDB Potato dextrose broth

QTL Quantitative trait loci

RAPD Random amplified polymorphic DNA

SNA Synthetic nutrient agar

SNB Synthetic nutrient broth

USWBSI U. S. Wheat and Barley Scab Initiative